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1956 Iodine Research Award Nominations Requested Prior to January 1, 1956

Nominations are now being received by the AMERICAN PHARMACEUTICAL ASSOCIATION for the 1956 Chilean Iodine Educational Bureau, Inc. Award recognizing outstanding research in the chemistry and pharmacy of iodine and its compounds as applied in pharmacy or medicine.

The award consists of $1,000 and a diploma setting forth the reasons for selection of the recipient. It may be presented each year at the annual meeting of the ASSOCIATION.

Any member of the ASSOCIATION may propose a nominee by submitting eight copies of each of the publications to be considered in the competition, a biographical sketch of the nominee including date of birth, and a list of his publications. Eight copies of the nomination must be submitted to Robert P. Fischelis, Secretary of the AMERICAN PHARMACEUTICAL ASSOCIATION, 2215 Constitution Ave., N. W., Washington 7, D. C. To be eligible for the 1955 Award, *nominations must be received on or before January 1, 1956*.

A nominee must be a resident of the United States or Canada. He must have accomplished outstanding research in the chemistry or pharmacy of iodine and its compounds as applied in pharmacy or medicine.

During the period covered by the nomination the nominee shall have been actively engaged in, shall have completed, or shall have published a report upon the line of investigation for which the award is made. During the period of two years prior to the date of nomination, the nominee shall not have been engaged in research under the sponsorship of the Chilean Iodine Educational Bureau, Inc.

The recipient will be selected by an award committee which is appointed by the chairman of the ASSOCIATION'S Council. The present committee includes Justin L. Powers, chairman; F. F. Blicke; Paul Block, E. A. Brecht, Stanley G. Mittelstaedt; Lloyd M. Parks, and Linwood F. Tice.

The recipient will deliver a paper or lecture upon the subject of his scientific work at the meeting at which the award is conferred. His paper, or address, will then be published in the JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION. Travel expense to the meeting at which the award is to be made is provided for up to $250.

The award is now in its eighth year. The recipient of the first award was Dr. William T. Salter, Yale University pharmacologist; the second recipient was Dr. George M. Curtis, Chairman of the Department of Surgical Research and Professor of Surgery at Ohio State University, and the third was Dr. C. P. Leblond, Professor of Anatomy, McGill University, Montreal, Canada. The fourth award was shared jointly by Dr. George Moore of the University of Minnesota Medical School and Dr. Moses Ashkenazy, Chief of Neurosurgery, Veterans Hospital, Houston, Tex. Dr. Veron H. Wallingford, Mallinckrodt Chemical Works, St. Louis, Missouri, was the recipient of the fifth award. Dr. Jack Gross, Associate Professor of Anatomy, State University of New York, College of Medicine in Brooklyn received the sixth award and Dr. Dominick Papa, Schering Corporation, Bloomfield, N. J., received the seventh award which was conferred during the 1955 Convention of the AMERICAN PHARMACEUTICAL ASSOCIATION.

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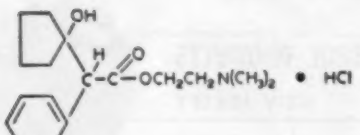
CYCLOPENTOLATE HYDROCHLORIDE.—Cyclogyl Hydrochloride.—$C_{17}H_{25}NO_3 \cdot HCl$.—M.W. 327.84.—β-Dimethylaminoethyl (1-hydroxycyclopentyl)-phenylacetate hydrochloride. The structural formula of cyclopentolate hydrochloride may be represented as follows:

Cyclopentolate Hydrochloride



Actions and Uses.—Cyclopentolate hydrochloride, a synthetic spasmolytic agent, produces a rapid, intense cycloplegia and mydriasis of moderate duration when instilled in the eye. Therefore, it is primarily useful for refraction studies and is effective in highly pigmented irises and for persons of all ages. The drug is also useful as a mydriatic in the management of iritis, iridocyclitis, keratitis, and choroiditis. For the prevention of lenticular adhesions, or, in conjunction with the use of miotics, it can be used for breaking or preventing adhesions formed during and after infections. No significant variation of intraocular tension has been reported from its use, but it is considered advisable to neutralize any cycloplegic in older patients in whom early, unrecognized glaucomatous changes may be present.

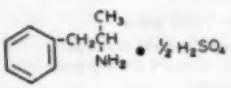
Cyclopentolate hydrochloride in solution does not produce any undesirable local or systemic effects after repeated instillation into the eye. It apparently is relatively nonirritating and nonsensitizing during local application. Results of studies with experimental animals indicate that it has a low toxicity and exhibits about one-half the antispasmodic activity of atropine. As with other cycloplegic-mydriatic agents, caution should be observed in patients with high intraocular pressure.

Dosage.—Cyclopentolate hydrochloride is administered only in the form of ophthalmic solutions for instillation into the conjunctival sac. For refraction in Caucasians, a dose of two drops of a 0.5% solution in each eye (each drop instilled at 5-minute intervals) for adults produces maximal cycloplegia in 30 to 60 minutes. Complete recovery occurs within 24 hours. The administration of one or two drops of 1 to 2% pilocarpine nitrate reduces recovery time to 6 hours or less. In deeply pigmented eyes of dark-skinned persons, satisfactory cycloplegia may be obtained with the 0.5% solution in about two-thirds of the cases. A 1% solution usually produces maximal cycloplegia in Negro patients; instillation of a 2% solution results in return of reading ability in 6 hours. For children, pretreatment with cyclopentolate on the day prior to examination is not usually necessary. Normally one or two drops of a 0.5 or 1% solution is instilled in each eye at the time of refraction, followed 10 minutes later by a second such application. This regime will produce satisfactory cycloplegia in all but the most refractory cases. If pretreatment in such individuals seems desirable one or two drops of 1% cyclopentolate may be instilled the evening prior to examination. Only in children with extremely dark irises has pretreatment with atropine been occasionally necessary.

For producing paralysis of the sphincter to correct spasm caused by inflammation, one or two drops of a 0.5% solution is instilled every 6 to 8 hours. For breaking or preventing lenticular adhesions secondary to infections, one or two drops of a 0.5% solution is instilled, followed in 6 hours by the instillation of 2% pilocarpine nitrate. Such alternate treatment should be carried out every 24 hours.

DEXTRO AMPHETAMINE SULFATE, U. S. P.—$(C_9H_{13}N)_2 \cdot H_2SO_4$.—M.W. 368.50.—*d*-1-Phenyl-2-aminopropane sulfate.—The dextrorotatory isomer of amphetamine sulfate.—The structural formula of dextro amphetamine sulfate may be represented as follows:

Dextro Amphetamine Sulfate U.S.P.



Actions and Uses.—Dextro amphetamine sulfate has the same actions and uses as the racemic compound, amphetamine sulfate, but exerts a predominantly greater stimulating effect on the central nervous system. Because of its relatively weak peripheral activity, it is generally regarded as less toxic than previously introduced sympathomimetic amines that are commonly employed clinically. Thus, it seldom gives rise to undesirable side-effects such as mydriasis, tremor, tachycardia, and changes in blood pressure. Dextro amphetamine sulfate, therefore, is useful by oral administration for the treatment of narcolepsy and postencephalitic parkinsonism, and as an adjunct in the management of acute and chronic alcoholism and alcoholic psychoses of recent origin. It is employed also for the symptomatic treatment of depressive states, especially to elevate the mood in early, mild, psychogenic depression characterized by apathy and psychomotor retardation and, to a variable or lesser extent, in psychoneuroses, and in severe depressions involving certain major psychopathic conditions of institutionalized patients. The drug also may be used as a stimulant in the management

of certain behavior problems of children, but it is not useful in schizophrenics and has an unfavorable effect on children with psychopathic personalities. The appetite-depressant effect of the drug also is useful as an adjunct in the dietary management of obesity.

Dextro amphetamine sulfate should not be employed as a stimulant by normal persons to mask fatigue caused by physical exertion or overwork. It should be used with caution in patients hypersensitive to sympathomimetic amines, those with coronary or cardiovascular disease, and those with severe hypertension. It is contraindicated in the presence of hyperexcitability and agitated prepsychotic states. If administered too late in the day, the drug may interfere with sleep.

Dosage.—Dextro amphetamine sulfate is administered orally. In the treatment of depressive states or alcoholism, the usual daily dosage ranges from 5 to 15 mg., administered as ordinary tablet or liquid medication in two or three doses at intervals of either 4 or 6 hours. The initial dose should be given on awakening so as to complete the total daily amount early in the day. For narcolepsy, the usual daily dosage for adults ranges from 10 to 50 mg., preferably in divided amounts; for postencephalitic parkinsonism, the daily dosage is usually 10 to 25 mg., also in divided amounts. To control appetite in obesity, the usual daily dosage for adults is 15 to 30 mg. in three divided doses, taken 30 to 60 minutes before meals. Light sleepers may take the final dose early (4 p. m.).

For children with behavior problems, the suggested dosage is 5 to 10 mg. in the morning and 2.5 to 5 mg. at noon. In all cases, the dosage should be individualized; it is advisable to begin with an initial dose of 5 mg. for adults or 2.5 mg. for children, followed by either one or two additional doses of the same amount. Dosage then can be increased to obtain the desired effect; the initial daily dose may be increased at first, leaving the repeated doses at the original level so that the major quantity is taken during the first half of the day. If necessary, the later doses may be equalized gradually to provide a uniform action.

A capsule containing 10 or 15 mg. of dextro amphetamine incorporated into variably coated pellets that afford continuous release of the drug over a period of 8 to 10 hours, thus prolonging the therapeutic effect for 10 to 12 hours, may be administered once daily in the morning to adults in place of ordinary oral medication in divided amounts.

ERYTHROMYCIN ETHYL CARBONATE.—Ilotycin ethyl carbonate.—Erythromycin ethyl carbonate is the ethyl carbonate ester of erythromycin, an antibacterial substance produced by the growth of *Streptomyces erythreus* Waksman. The structural formula of erythromycin ethyl carbonate has not been established.

Actions and Uses.—Erythromycin ethyl carbonate, a salt of erythromycin, shares the actions and uses of the parent antibiotic. (See New and Nonofficial Remedies 1955, under the monograph on erythromycin.) The ethyl carbonate salt is suitable for the extemporaneous preparation of flavored suspensions of the drug for oral administration.

Dosage.—Erythromycin ethyl carbonate is administered orally in doses expressed in terms of erythromycin base. For adults, a dose equivalent to 200 mg. of erythromycin every 4 to 6 hours is considered adequate. The optimal dosage for children has not been finally determined, but a dose equivalent to 5 mg. of erythromycin per lb. of body weight administered every 6 hours is considered reasonable.

LENTE INSULIN.—"Lente insulin is a sterile suspension, in a buffered water medium, of insulin modified by the addition of zinc chloride. Of the insulin contained in the preparation not more than 1 U. S. P. Unit of insulin per milliliter is in solution, approximately 70 per cent is crystalline, and the remainder is amorphous. Zincinsulin crystals are used in such quantity that each milliliter of the preparation, when the precipitate therein is brought into uniform suspension, contains either 40 or 80 U. S. P. Units of insulin. The preparation contains, for each 100 U. S. P. Units of insulin not less than 0.20 milligram and not more than 0.25 milligram zinc (of which not less than 40 per cent not more than 65 per cent is in the supernatant liquid), and not more than 0.65 milligram nitrogen. The preparation also contains not less than 0.15 per cent and not more than 0.17 per cent (w/v) sodium acetate, not less than 0.65 per cent and not more than 0.75 per cent (w/v) sodium chloride, and not less than 0.09 per cent and not more than 0.11 per cent (w/v) methyl-*p*-hydroxybenzoate. The *p*H of the finished product is not less than 7.1 not more than 7.5." Certification of Batches of Drugs Composed Wholly or Partly of Insulin (19 Fed. Reg. 4153 [July 8] 1954).

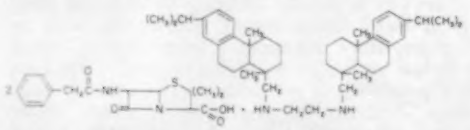
Actions and Uses.—Lente insulin is a mixture of minute particles consisting of approximately 70% crystalline zinc insulin and 30% amorphous zinc insulin, each component of which has a sufficiently high zinc content to make the mixture relatively insoluble at the *p*H of the blood. The proportion of the components provides an antidiabetic action that is intermediate in time between that of unmodified (regular) insulin and protamine zinc insulin. The time of action of lente insulin so closely approximates that of the modified protamine zinc insulin, isophane (NPH) insulin, that they can be used interchangeably. The onset of action of lente insulin is sufficiently prompt to control the after breakfast rise in blood sugar, and its duration of activity is usually adequate to maintain blood sugar levels during fasting without the need for supplementary injections. Lente insulin, which contains no foreign modifying protein, is free from the possibility of producing local sensitivity reactions attributed to protamine or globin. Since lente insulin differs clinically from unmodified insulin only in its more prolonged action in lowering the blood sugar, its administration and dosage should follow the same principles that govern the use of insulin in general; however, lente insulin is not adaptable for use in place of unmodified insulin in dealing with diabetic emergencies that require immediate-acting intravenous insulin. (Also see New and Nonofficial Remedies 1955, under the general statement on insulin.)

Dosage.—Lente insulin is administered as a buffered suspension by deep subcutaneous injection. It should not be injected into underlying muscle and is never administered intravenously.

The container vial should be rotated and inverted several times to insure uniform distribution of the suspended particles, but vigorous shaking and frothing should be avoided. The potency is expressed in terms of insulin units per cubic centimeter of suspension. The number and size of daily doses, time of administration, diet, and exercise must be determined by careful observation under laboratory control, with frequent blood sugar estimations and urinary sugar examinations in each individual case. Usually the most satisfactory time for injection is in the morning before breakfast. In newly developed, uncomplicated cases of average severity, an initial daily dose of 10 units may be administered before breakfast; this may then be increased by 3 to 5 units until proper control of blood and urinary sugar is achieved. In patients already under treatment with protamine zinc insulin or unmodified insulin or both, a beginning dose of lente insulin of approximately 20% fewer units may be substituted; this is then increased if necessary. Patients on isophane insulin may be transferred directly to lente insulin on a unit-for-unit basis. In certain severe cases, further regulation of diet may be important to obtain the optimum dosage level. As with protamine zinc insulin, the proportional distribution of food of one-fifth, two-fifths, and two-fifths respectively for the three meals may be advantageous. Afternoon and bedtime feedings may become desirable or necessary in some circumstances at the discretion of the physician.

Suspensions of lente insulin should be stored in a cold place, preferably a refrigerator, Exposure to freezing or high temperatures should be avoided. Vials in use also should be protected from strong light and the contents used as continuously as possible. A partially empty vial not used for several weeks should be discarded. A vial in which the precipitated suspension has become clumped or deposited on the wall of the container should not be used.

HYDRABAMINE PENICILLIN G.—Hydrabamine penicillin G is a mixture of crystalline penicillin G salts consisting chiefly of the salt of N,N′-bis-(dehydroabietyl)ethylenediamine, with smaller amounts of the salts of the dihydro- and tetrahydro-derivatives. The structural formula of hydrabamine penicillin G may be represented as follows:



Actions and Uses.—Hydrabamine penicillin G, a water-insoluble dipenicillin compound salt of a rosin amine base, is useful in the treatment and prevention of penicillin-susceptible infections. (See New and Nonofficial Remedies 1955, under the general statement on penicillin). Its prophylactic use should be limited to streptococcic and pneumococcic infections of the throat and ears, to procedures such as tonsillectomy and dental extractions, and to patients with a history of rheumatic fever or rheumatic heart disease. As with other penicillin compounds, it is of no value for the treatment or prevention of the common cold or influenza.

Given orally, hydrabamine penicillin G promptly produces satisfactory penicillin blood levels when adminstered in doses of 300,000 to 600,000 units at at 6-hour intervals. After 6 hours, the blood level produced by similar single oral doses falls rapidly. The onset of the maximum blood level produced by single oral doses is only slightly delayed when administered with meals and the height and duration of the blood level is not affected appreciably by digestive function. Median blood levels obtained following oral adminstration of 600,000 unit doses are approximately twice those obtained with 300,000 unit doses. Animal studies indicate that the hydrabamine base portion of the compound is chiefly unabsorbed when given by the oral route; up to 90% is recovered in the feces with less than 1% found in the urine. Fecal excretion of the base is not significantly influenced by the normal intestinal flora.

Hydrabamine penicillin G has about the same toxicity in animals as benzathine penicillin G. Chronic toxicity studies in animals with doses far in excess of those recommended for human beings have not disclosed hematological or other abnormalities. Clinically, looseness of stools has been observed as a rare side-effect and urticaria has been attributed to the drug. Physicians should be alert to the possibility of allergic reactions, stomatitis, or monilial infection of the gastrointestinal tract. As with other penicillin compounds, allergic reactions may be controlled with antihistamine medication, but when this fails or when there are monilial complications, the drug should be discontinued.

Dosage.—Hydrabamine penicillin G is administered orally for acute infections in initial doses of 300,000 to 600,000 units every 6 hours for adults; subsequently dosage should be regulated according to the therapeutic response. In children, oral doses of 300,000 to 600,000 units four times daily may be adequate. To maintain continuous prophylaxis in rheumatic fever, approximately 300,000 units orally once or twice daily is suggested; patients with streptococcic infections who have had rheumatic fever or show signs of rheumatic heart disease should receive a total of 800,000 to 1,200,000 units per day (a smaller amount for children, larger for adults) in four divided doses for the first 5 days, and 600,000 to 750,000 units per day in three divided doses for the second 5 days. The entire 10 day treatment should be administered to such patients even though fever or symptoms disappear before the end of this period.

POLIOMYELITIS IMMUNE GLOBULIN (HUMAN).—Poliomyelitis immune globulin (human is a sterile solution of globulins that contains those antibodies normally present in adult human blood. Each lot is derived from an original plasma or serum pool that represents at least 1,000 individuals and is processed to contain 165 mg. (±15 mg.) of globulin per cubic centimeter. It is standardized for its content of poliomyelitis antibodies in compliance with the requirements of the National Institutes of Health, of the United States Public Health Service, including the release of each lot individually before its distribution.

Actions and Uses.—Poliomyelitis immune globulin (human) is a passive immunologic agent that contains significant concentrations of antibodies useful for the attenuation or prevention of poliomyelitis,

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N. N. R.

. . . . . *from page VI*

measles, and infectious (epidemic) hepatitis. Significant but temporary protection against the development of paralytic poliomyelitis has been produced from the second through the fifth week following injection; but the likelihood of securing protection against paralytic poliomyelitis by the use of the globulin is much less than the likelihood of securing protection against measles or infectious (epidemic) hepatitis by the same means. The preparation is equivalent in usefulness to immune serum globulin (human) for the prevention or modification of measles when injected within the first 6 days, but not beyond the 10th day, following initial exposure. It also prevents or attenuates infectious (epidemic) hepatitis when injected during the incubation period; it apparently confers passive immunity to that infection for 6 to 8 weeks.

Poliomyelitis immune globuline (human) is regarded as being free from the virus of serum hepatitis. Sensitization to repeated injections is extremely rare. Injections occasionally may be followed by local tenderness and stiffness of muscles persisting for several hours. Care should be exercised to avoid accidental intravenous administration.

Dosage.—Poliomyelitis immune globulin (human) is administered only by intramuscular injection, preferably in the buttock. *Careful technique is essential to avoid accidental intravenous injection.*

For protection against paralytic poliomyelitis, the average dose to be injected is calculated on the basis of 0.14 cc. per lb. (0.31 cc. per Kg.) of body weight. This dose may be repeated in 6 weeks if continued protection is desirable.

For the modification of measles, the dose is calculated on the basis of 0.02 to 0.0246 cc. per lb. (0.044 to 0.055 cc. per Kg.) of body weight; for complete prevention, at least 0.10 cc. per lb. (0.22 cc. per Kg.) should be employed. Either dose preferably should be given within 6 days after initial exposure.

For prevention or attenuation of infectious (epidemic) hepatitis, the suggested dose is 0.02 cc. per pound (0.044 cc. per kilogram) of body weight in children or adults; this dose should be repeated in 3 weeks if longer protection is desired.

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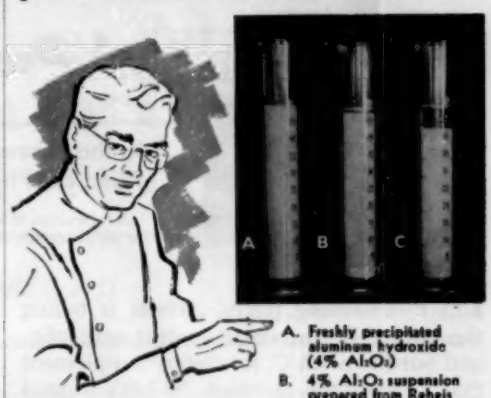

A. Freshly precipitated aluminum hydroxide (4% Al_2O_3)
B. 4% Al_2O_3 suspension prepared from Reheis F-1000
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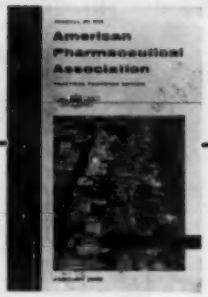

A Preview of Advertising

In the November 1955 issue of the Practical Pharmacy Edition

(All references to actions and uses are taken from the statements of the producers of the products mentioned.)

▶ In three *selling* pages, **Abbott** is telling the story of **Erythrocin,** its potent antibiotic, and **Sur-Bex with C,** its dietary supplement that answers the question, "What's a *good* B complex?"

▶ Dominating **Hoffmann-La Roche's** presentation is an umbrella connoting the seasonal protection afforded by **Romilar Expectorant,** non-narcotic cough specific.

▶ The **Hynson, Westcott & Dunning** announcement points up its antiseptic, *Merodicein,* and *Saligenin,* a mild local anesthetic, which combine to make **Thantis Lozenges** "first aid for throat irritations."

▶ **Lancet, Ltd.,** manufacturers' representatives, is interested in handling additional products available for sale throughout eastern Canada to pharmacies, physicians, hospitals and religious institutions.

▶ **Achromycin** *Nasal Suspension* is the subject of **Lederle's** current colorful insertion.

▶ *New* **'Ilotycin,' I.M.** now makes possible the intramuscular administration of this antibiotic of **Eli Lilly and Company.**

▶ Said to be "virtually without side effects," is **Salcort,** a new antiarthritic compounded by **The S. E. Massengill Company.**

▶ *Newly offered* by **Merck & Co.** is an inverted Rx Display Set of 8-ounce fused-label bottles designed to add colorful interest to the Rx department.

▶ *White* molded closures "seal (**Owens-Illinois**) **Duraglas** Rx containers professionally . . . efficiently."

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▶ "Now," says the advertiser, "two ready-mixed dosage forms of **Pfizer**-discovered tetracycline—*new* **Tetra Bon** and *new* (vitamin-fortified) **Tetra Bon SF.**"

▶ The **A. H. Robins Co.** delineates the efficacy of **Pabalate-HC,** plus its safety and economy, in treating rheumatic affections.

▶ **Stimavite** *Tastitabs* are **Roerig-***recommended* to stimulate the appetites; growth of youngsters; no less effective in meeting the problems of oldsters is Roerig's "5-factor geriatric supplement, **Neobon.**"

▶ To "prevent reactions—protect your penicillin therapy . . ." **Schering** suggests adding **Chlor-Trimeton** to aqueous penicillin.

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Scientific Edition

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLIV — *NOVEMBER 1955* — NUMBER 11

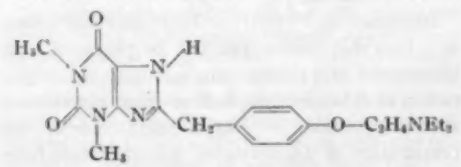
CONSECUTIVE No. 12

Arylketones and Thiomorpholides in the Synthesis of 8-Substituted-Xanthines*

By GEORGE P. HAGER and STANLEY P. KRAMER

The preparation of 8-aralkyl-theophyllines and related xanthines by reaction of 1,3-dimethyl-5,6-diaminouracil with appropriate carboxylic acids has been reported THIS JOURNAL, 43, 152(1954). The extension of this work to include 8-aralkyl-theophyllines substituted in the aralkyl group involved the use of substituted phenylacetic acids, some of which were obtained from arylketones by way of thiomorpholides using the Willgerodt reaction. Both the arylketones and the thiomorpholide intermediates in the Willgerodt reaction have been found to react with 1,3-dimethyl-5,6-diaminouracil under the proper conditions to produce the 8-substituted-theophyllines.

THE PHARMACOLOGIC PROPERTIES of 8-aralkyl-theophyllines indicated that substitution of the aralkyl group with an electron-withdrawing group (sulfo group) was detrimental to vasodepressor activity, while similar substitution with an electron-releasing group (amino group) appeared to enhance such activity (1). The presence in these compounds of a polar group, preferably one through which salts stable in aqueous solution could be formed, appeared desirable from the standpoint of administration and absorption. It was hoped that both of the possible effects of an electron-releasing polar substituent, *viz.*, enhancement of activity and solubilization through salt formation, could be realized in the salts of 8-[4-(2-diethylaminoethoxy)-benzyl]-theophylline (I).



I

The methiodide of (I) was found to form stable aqueous solutions; however, on intravenous administration to dogs under ether anesthesia, only a transient fall in blood pressure occurred accompanied by respiratory depression and paralysis.[1] The related compound, 7-(2-diethylaminoethyl)-8-[4-(2-diethylaminoethoxy)-benzyl]-theophylline dihydrochloride, exhibited similar pharmacologic behavior.

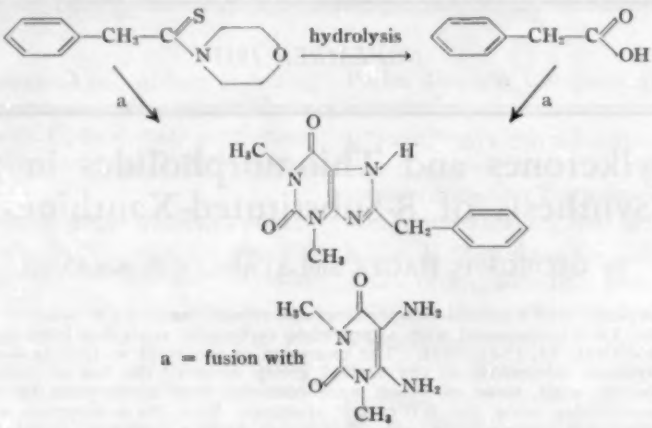
A series of mono- and dihydroxybenzyl-theophyllines and corresponding ethers was also prepared in order to study further the effect of electron-releasing groups on activity which was evident in the analogous 8-(4-aminobenzyl)-theophylline (1). The compound, 8-(α-methoxybenzyl)-theophylline, was included for comparison with related compounds having the methoxyl group substituted in the ring. For the preparation of 8-aralkyl-theophyllines, the Traube method (2) described for theophylline, has generally been employed (1, 3–5).

In the synthesis of the hydroxy-substituted-benzyltheophyllines, the intermediate substituted phenylacetic acids were frequently obtained from the corresponding ketones by the Will-

* Received May 5, 1955, from the School of Pharmacy, University of Maryland, Baltimore.
Presented to the Scientific Section, A. PH. A., Miami Beach meeting, May 1955.

[1] The authors acknowledge with appreciation the assistance of Dr. John C. Krantz, Jr., in the pharmacologic studies herein reported.

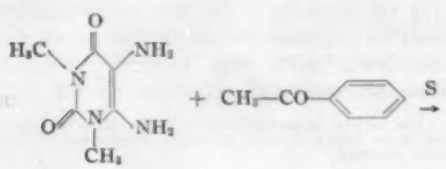
gerodt reaction. In order to avoid the time-consuming hydrolysis of the thiomorpholide intermediates, fusion of the thiomorpholide with 1,3-dimethyl-5,6-diaminouracil, a reaction analogous to the cleavage of N-substituted-phthalimides with hydrazine hydrate (6), was studied. 8-Benzyltheophylline was produced in this manner from phenylthioacetomorpholide and 1,3-dimethyl-5,6-diaminouracil in yields of about 30 per cent (Eq. 1).



(1)

It is especially desirable to avoid the hydrolysis of the thiomorpholides, which would serve as intermediates in the preparation of hydroxy- and alkoxy - substituted - 8- benzyltheophyllines, because of the instability of polyhydric phenols in alkaline medium and the tendency of phenolic ethers to undergo fission in acid medium. The preparation of purines by the reaction of amides with diaminouracils has been described (7) as well as the preparation of 2-substituted-2-imidazolines, such as 2-benzyl-2-imidazoline, by reaction of thioamides with ethylenediamine (8).

Since 2-substituted-imidazoles, hence 8-substituted-xanthines, may be considered as derivatives, cyclic amidines, of carboxylic acids (9), and since the Willgerodt serves to produce derivatives of carboxylic acids, such as thiomorpholides, a logical extension of this investigation was the attempt to prepare 8-aralkyl-theophyllines directly from ketones by the use of 1,3-dimethyl-5,6-diaminouracil in place of the usual basic component (morpholine, etc.) in the Willgerodt reaction (Eq. 2).



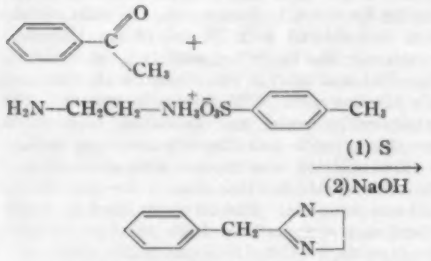
(2)

The reaction of an excess of acetophenone with 1,3-dimethyl-5,6-diaminouracil in the presence of sulfur produced 8-benzyltheophylline in yields of 29 to 30 per cent. The yields from the ketone and from the thiomorpholide were about the same, but the product obtained from the thiomorpholide seemed to require less purification.

The use of trithioacetophenone in place of acetophenone in the Kindler modification of the Willgerodt reaction was reported to improve the yield of phenylthioacetomorpholide (10). When trithioacetophenone was used in place of acetophenone in a reaction with 1,3-dimethyl-5,6-diaminouracil and sulfur, much lower yields of 8-benzyltheophylline were obtained. Styrene has also been employed successfully in place of acetophenone for the production of amides by the Willgerodt reaction (11); however, use of styrene in place of acetophenone in reaction with 1,3-dimethyl-5,6-diaminouracil and sulfur, resulted in a product from which no 8-benzyltheophylline could be isolated.

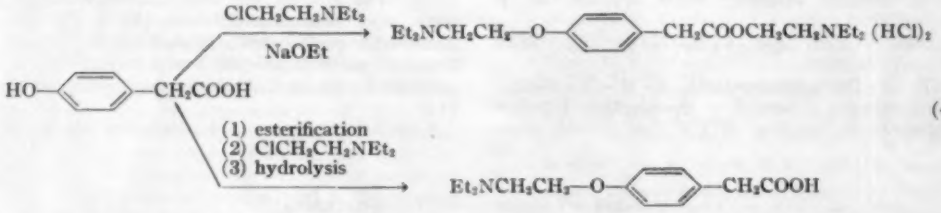
Inasmuch as 2-substituted-2-imidazolines, such as 2-benzyl-2-imidazoline, can be prepared from thioamides in a manner analogous to the preparation of 8-benzyltheophylline from phenylthioacetomorpholide (8), an investigation of the preparation of 2-substituted-2-imidazolines from ketones similarly to the preparation of 8-benzyltheophylline directly from ketones was undertaken. The reaction of acetophenone with ethylenediamine *p*-toluenesulfonate in the pres-

ence of sulfur has been found to produce 2-benzyl-2-imidazoline in yields of 3 to 6 per cent under the conditions which have thus far been employed (Eq. 3). A further study of the reaction with the objective of improving yields is being carried out.



(3)

4-(2-Diethylaminoethoxy)-phenylacetic acid, required for the preparation of compound I, was prepared from 4-hydroxyphenylacetic acid by way of ethyl 4-hydroxyphenylacetate and ethyl 4-(2-diethylaminoethoxy)-phenylacetate. The reaction of 4-hydroxyphenylacetic acid with N-(2-chloroethyl)-diethylamine in a mixture of sodium ethoxide and dimethylformamide produced 2-diethylaminoethyl 4-(2-diethylaminoethoxy)-phenylacetate, which was isolated as the dihydrochloride. These reactions are summarized in Eq. 4.



(4)

The pharmacologic properties of the substituted benzoates (12) corresponding to the substituted phenylacetates indicated in Eq. 4, were of sufficient interest to warrant further study of the compounds of this series.

EXPERIMENTAL

All melting points were obtained by use of a Fisher-Johns melting point apparatus.

Substituted Phenylacetic Acids.—The Willgerodt reaction using the appropriate ketone was employed for preparation of 4-methoxy- (13–15), 4-ethoxy-, and 4-benzyloxyphenylacetic acids (16). 3,4-Methylenedioxyphenylacetic acid was prepared from piperonal by way of the azlactone and the phenylpyruvic acid (17). α-Methoxytoluic acid was prepared by methylation of mandelic acid (18). 3-Hydroxy-, 4-hydroxy-, and 3,4-dihydroxyphenylacetic acids were obtained by acid cleavage of the corresponding methoxy-derivatives (19). The other substituted-phenylacetic acids, with the exception of 4-(2-diethylaminoethoxy)-phenylacetic acid, were commercially available.

Ethyl 4-(2-Diethylaminoethoxy)-phenylacetate.—Ethyl 4-hydroxyphenylacetate was prepared by esterification of 4-hydroxyphenylacetic acid in the usual way; b_2 147°; reported $b_{760.5}$ 314° corr. (20).

A mixture of 44.0 Gm. (0.26 mole) of N-(2-chloroethyl)-diethylammonium chloride, 22.5 Gm. (0.13 mole) of ethyl 4-hydroxyphenylacetate, 138.0 Gm. of anhydrous potassium carbonate, and 754 ml. of dry acetone was refluxed while stirring for fourteen hours. After filtering, the solvent was removed by distillation under reduced pressure. The residue was extracted with ether and the ether solution was washed with 10% sodium hydroxide and water and dried using anhydrous magnesium sulfate. The ether was removed by distillation and the residue, distilled under reduced pressure, gave a light yellow oil, b_2 155–164°; 20.0 Gm. (57%). For analysis, a sample was redistilled, b_1 155–156°.

Anal.—Calcd. for $C_{16}H_{25}NO_3$: N, 5.01. Found: N, 4.84.

The hydrochloride was prepared in the usual way and recrystallized from a mixture of ethanol and ether; white crystals, m. p. 131.5–132.5°.

Anal.—Calcd. for $C_{16}H_{26}ClNO_3$: N, 4.44. Found: N, 4.39.

4-(2-Diethylaminoethoxy)-phenylacetic Acid Hydrochloride.—A mixture of 6.0 Gm. (0.02 mole) of ethyl 4-(2-diethylaminoethoxy)-phenylacetate, 5 ml. of hydrochloric acid, and 40 ml. of water was refluxed for eight hours. The product was evaporated under reduced pressure to a thick syrup which was rubbed with acetone to produce a white crystalline solid. After filtration and washing with acetone and ether, 5.5 Gm. (89%) of product was obtained; m. p. 127.0–128.5°.

Anal.—Calcd. for $C_{14}H_{22}ClNO_3$: N, 4.95. Found: N, 5.02.

2-Diethylaminoethyl 4-(2-Diethylaminoethoxy)-phenylacetate Dihydrochloride.—4-Hydroxyphenylacetic acid (15.2 Gm., 0.1 mole) was added to a solution of 13.6 Gm. of sodium ethoxide in 75 ml. of absolute ethanol, and the solvent was removed by distillation under reduced pressure. The residue was suspended in 125 ml. of dimethylformamide and 67.8 Gm. (0.25 mole) of freshly distilled N-(2-chloroethyl)-diethylamine was added. After refluxing the mixture for five hours and filtering, the solvent was removed by distillation under reduced pressure and the product, a brown oil, was collected at 190–211°/2 mm. The dihydrochloride was prepared in the usual way and recrystallized from a mixture of ethanol and ether; white crystals, 4.8 Gm. (10%), m. p. 158–159°.

Anal.—Calcd. for $C_{20}H_{36}Cl_2N_2O_3$: N, 6.62. Found: N, 6.60.

Preparation of 8-Substituted-theophyllines.—An intimate mixture of equimolecular amounts of the appropriate phenylacetic acid and 1,3-dimethyl-5,6-diaminouracil was heated with occasional stirring in a beaker immersed in an oil bath. The bath temperature was gradually raised until the mixture fused and was then held at a slightly higher temperature until it resolidified (Table I). The mixture was dissolved in excess 5% sodium hydroxide and the solution was heated at its boiling point for about thirty minutes. The solution was cooled, filtered, and saturated with carbon dioxide to precipitate the 8-substituted-theophylline. The product was purified by recrystallization, using decolorizing carbon as necessary, from glacial acetic acid, absolute ethanol, dimethylformamide, or mixtures of acetic acid with water, ethanol, or ether. The 8-substituted-theophyllines prepared as above indicated, and their properties, are summarized in Table I.

8 - [4 - (2 - Diethylaminoethoxy) - benzyl] - theophylline Hydrochloride and Methiodide.—By treatment of a suspension of 8-[4-(2-diethylaminoethoxy)-benzyl]-theophylline in absolute ethanol with a solution of hydrogen chloride in absolute ethanol until the solution was acid on moist congo red paper, followed by addition of ether, the white crystalline hydrochloride was obtained. This was recrystallized repeatedly from a mixture of absolute ethanol and ether and finally from absolute ethanol; m. p. 193.4–194.5°.

Anal.—Calcd. for $C_{20}H_{28}ClN_5O_3$: N, 16.60. Found: N, 16.40.

The methiodide was prepared by reaction of 8-[4-(2-diethylaminoethoxy)-benzyl]-theophylline with methyl iodide in absolute ethanol, and recrystallized from absolute ethanol; white crystals, m. p. 207–208°.

Anal.—Calcd. for $C_{21}H_{30}IN_5O_3$: N, 13.28. Found: N, 13.16.

7 - (2 - Diethylaminoethyl) - 8 - [4 - (2 - diethylaminoethoxy) - benzyl] - theophylline Dihydrochloride.—A mixture of 0.8 Gm. (0.034 gram-atom) of sodium, 5 ml. of absolute ethanol, and 75 ml. of xylene was refluxed until the sodium dissolved. 8-(4-Hydroxybenzyl)-theophylline (4.8 Gm., 0.02 mole) was added and 8 ml. of solvent was distilled off to remove most of the ethanol. To the mixture (5.7 Gm., 0.042 mole) of freshly distilled N-(2-chloroethyl)-diethylamine dissolved in 30 ml. of xylene was added during thirty minutes. After refluxing for three to four hours, the mixture was cooled and diluted with 75 ml. of ethyl acetate. The mixture was extracted with 10% hydrochloric acid. The acid extract was washed with ether and made alkaline with 10% sodium hydroxide. The precipitated material was extracted with ether. After drying with anhydrous magnesium sulfate, the ether solution was treated with a solution of hydrogen chloride in ethanol until precipitation of an oil was complete. The oil crystallized on standing and was recrystallized from absolute ethanol; white crystals, 1.5 Gm. (16%), m. p. 232–234°.

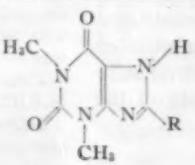
Anal.—Calcd. for $C_{28}H_{42}Cl_2N_6O_3$: N, 15.07. Found: N. 15.22.

Preparation of 8-Substituted-theophyllines from Thiomorpholides.—An intimate mixture of 7.4 Gm. (0.038 mole) of phenylthioacetomorpholide (15) and 5.7 Gm. (0.033 mole) of 1,3-dimethyl-5,6-diaminouracil was heated in a test tube immersed in an oil bath while stirring constantly. The bath temperature was gradually increased from 110 to 175° over a period of seven hours. After adding 75 ml. of 5% sodium hydroxide, the mixture was heated under reflux for one hour. After treatment with decolorizing carbon, the mixture was filtered, cooled, and saturated with carbon dioxide; and 3.8 Gm. of crude 8-benzyltheophylline precipitated. The product was recrystallized from 70% acetic acid, producing 2.9 Gm. (32%) of white needles, m. p. 297–298°, mixture with 8-benzyltheophylline (m. p. 287–289°) m. p. 294–298°.

Anal.—Calcd. for $C_{14}H_{14}N_4O_2$: N, 20.73. Found: 20.57.

A similar treatment of 4-aminophenylthioaceto-

TABLE I.—8-SUBSTITUTED-THEOPHYLLINES



Compound (R is given)	Temperature of reaction, °C.	M. p.	Formula	Analysis, % N Calcd.	Found	Yield, %
3-hydroxybenzyl-	160–75[a]	>300	$C_{14}H_{14}N_4O_3$	19.57	19.66	46
4-hydroxybenzyl-	160–75[b]	>300	$C_{14}H_{14}N_4O_3$	19.57	19.15	42
3,4-dihydroxybenzyl-	175–80[b]	>300	$C_{14}H_{14}N_4O_4$	18.53	18.31	14
3-methoxybenzyl-	130–40	251–252	$C_{15}H_{16}N_4O_3$	18.66	18.68	67
4-methoxybenzyl-	135–40	276.7–277.5	$C_{15}H_{16}N_4O_3$	18.66	18.48	51
4-ethoxybenzyl-	140–50	256	$C_{16}H_{18}N_4O_3$	17.83	17.75	69
4-benzyloxybenzyl-	145–55	235.5–257.0	$C_{21}H_{20}N_4O_3$	14.89	14.55	33
4-(2-diethylaminoethoxy)-benzyl-	130–97[c]	189.5–190.0	$C_{20}H_{27}N_5O_3$	18.17	17.89	64
3,4-dimethoxybenzyl-	125–35	246–247	$C_{16}H_{18}N_4O_4$	16.96	16.86	61
3,4-methylenedioxybenzyl-	135–45	>300	$C_{15}H_{14}N_4O_4$	17.86	17.70	54
α-methoxybenzyl-	135–40	193.5–194.0	$C_{15}H_{16}N_4O_3$	18.66	18.55	75

[a] Carried out in an evacuated system.
[b] Carried out in a nitrogen atmosphere.
[c] Mixture did not resolidify.

morpholide (15) resulted in a 5% yield of 8-(4-aminobenzyl)-theophylline, m. p. 297–298° decompn., reported m. p. 297–298° (1).

Increasing the proportion of phenylthioacetomorpholide or conducting the reaction in an evacuated system did not improve the yields.

Preparation of 8-Benzyltheophylline Directly from Acetophenone.—An intimate mixture of 7.2 Gm. (0.06 mole) of acetophenone, 2.4 Gm. (0.08 gram-atom) of sulfur, and 5.1 Gm. (0.03 mole) of 1,3-dimethyl-5,6-diaminouracil was heated under reflux with constant stirring, and the temperature was raised from 155 to 170° during thirty minutes, and maintained at 170° for six hours. Five per cent sodium hydroxide (60 ml.) was added and the mixture was refluxed for one hour. The cooled mixture was washed with ether and saturated with carbon dioxide; and, after washing the precipitate with ethanol and ether, 4.2 Gm. of product was obtained. The product was dissolved in 125 ml. of glacial acetic acid by heating, and the solution was filtered immediately after cooling to room temperature. The filtrate was evaporated almost to dryness under reduced pressure, and the residue was washed with water, alcohol, and, ether; white crystals, m. p. 276–278°, mixture with 8-benzyltheophylline (m. p. 287–289°), m. p. 282–284°. The product of the reaction can be further purified by recrystallization from 70% acetic acid.

Lower yields were obtained when the reaction was carried out at lower temperatures (138°), when lower proportions of acetophenone were employed, and when lower proportions of sulfur were used. When higher proportions of sulfur were used, the yield was not improved and the product was more difficult to purify.

When styrene or trithioacetophenone (21) were used in place of acetophenone under similar conditions, little or no 8-benzyltheophylline was obtained.

Preparation of 2-Benzyl-2-imidazoline from Acetophenone.—An intimate mixture of 23.2 Gm. (0.1 mole) of ethylenediamine *p*-toluenesulfonate, 3.2 Gm. (0.1 gram-atom) of sulfur, and 12.0 Gm. (0.1 mole) of acetophenone was heated under reflux in a flask immersed in an oil bath. The mixture fused when the bath temperature reached 120–125°, and the bath temperature was raised during one hour to 170° and held at 170–185° for ten hours. At the end of this reflux period, hydrogen sulfide evolution apparently ceased, and the mixture was treated with 200 ml. of 5% sodium hydroxide. The mixture was extracted with ether, and the ether solution was dried and concentrated by flash distillation of the ether. The residue was distilled under reduced pressure and a light yellow oil, solidifying readily at room temperature, was collected, b_3 134–145°. The product was treated with a solution of hydrogen chloride in absolute ethanol, and the resulting solution precipitated by addition of anhydrous ether. The crystalline solid was recrystallized from a mixture of ethanol and ether; 0.72 Gm. (3.7%), m. p. 171–173°, reported m. p. (2-benzyl-2-imidazolinium chloride), 174° (22). The picrate was prepared in the usual way, m. p. 147–149°, reported m. p. (2-benzyl-2-imidazoline picrate), 149° (22).

Current studies indicate that yields can be improved as more favorable reaction conditions are established. When ethylenediamine was used instead of ethylenediamine *p*-toluenesulfonate, no 2-benzyl-2-imidazoline could be isolated from the tarry product.

SUMMARY

1. The preparation of 8-benzyl- and 8-(4-aminobenzyl)-theophyllines from the corresponding phenylthioacetomorpholides is described.

2. The preparation of 8-benzyltheophylline and 2-benzyl-2-imidazoline from acetophenone is described.

3. A series of 8-hydroxybenzyl- and 8-dihydroxybenzyltheophyllines and certain of their ethers has been prepared.

4. The methiodide of 8-[4-(2-diethylaminoethoxy)-benzyl]-theophylline and the dihydrochloride of 7-(2-diethylaminoethyl)-8-[4-(2-diethylaminoethoxy)-benzyl]-theophylline, while soluble in water, produced only a transient lowering of the blood pressure accompanied by respiratory depression and paralysis.

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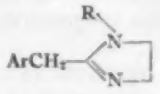
N,N-Dialkyl-dialkylenetriamines and Corresponding Imidazolines*

By GEORGE P. HAGER and HATIF H. AL-JALEEL

A convenient method has been found for the synthesis of N,N-dialkyl-diethylenetriamine and its homologs. These substances can be used in synthesis of 1-(N,N-dialkylaminoalkyl)-2-aralkyl-2-imidazolines which are of pharmacologic interest because of their structural relationship to 2-benzyl-2-imidazoline.

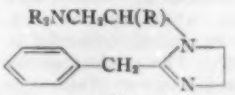
SUBSTITUTED IMIDAZOLINES exhibit a complex pattern of pharmacodynamic behavior, such that a precise classification by current standards is difficult. For example, 2-aralkyl-2-imidazolines (I) may be either vasoconstrictive (hypertensive) or vasodilating (hypotensive) depending on the nature of the aralkyl group ($ArCH_2$, formula I) and on the presence of other substituents (R, formula I) especially in the 1-position of the 2-imidazoline nucleus.



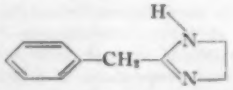
I

The fact that 2-(1-naphthylmethyl)-2-imidazoline (Ar = 1-naphthyl) and 2-(3,4,5-trimethoxybenzyl)-2-imidazoline (Ar = 3,4,5-trimethoxyphenyl) are hypertensive while 2-benzyl-2-imidazoline (Ar = phenyl) is hypotensive, indicates a surprising overlapping of vasomotoric properties dependent on the nature of the aralkyl group (1). The introduction of a methyl substituent in the 1-position of 2-aralkyl-2-imidazolines engenders a considerable change in certain pharmacodynamic properties as indicated by the hypertensive activity of 1-methyl-2-benzyl-2-imidazoline in contrast to the hypotensive activity of 2-benzyl-2-imidazoline. A comprehensive study of the pharmacodynamic properties of several 2-aralkyl-2-imidazolines and their 1-methyl-derivatives led to the conclusion that, regardless of the activity of the former (pressor or depressor), the latter all produce a rapid rise in blood pressure and increased heart rate. Whereas 2-benzyl-2-imidazoline produces a fall in blood pressure resulting from peripheral vasodilation, its 1-methyl derivative affects the blood pressure by a more central action involving stimulation of sympathetic ganglia, release of epinephrine by the adrenals, and direct action on heart muscle. Cholinergic and curariform activities under various conditions and at various sites are common to both types of compound (2, 3, 4).

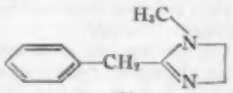
A series of 1-substituted-2-benzyl-2-imidazolines (II) has been prepared from N,N-disubstituted-dialkylenetriamines and these compounds bear a relationship to 2-benzyl-2-imidazoline (III) similar to that of 1-methyl-2-benzyl-2-imidazoline (IV).



II

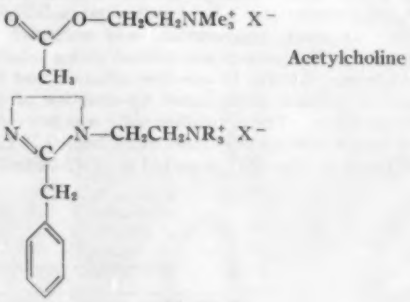


III



IV

It is anticipated that the aminoalkyl group substituted in the 1-position (II) will have an effect similar to that of a methyl group in the same position (IV) on the peripheral vasodilating properties of the compound. Quaternization of the extranuclear amine function increases structural similarity to acetylcholine (Fig. 1) so that the possible cholinergic and curariform activities of the 1-(disubstituted-aminoalkyl)-2-benzyl-2-imidazolines and their quaternary salts are considered worthy of investigation.



Acetylcholine

Figure 1.

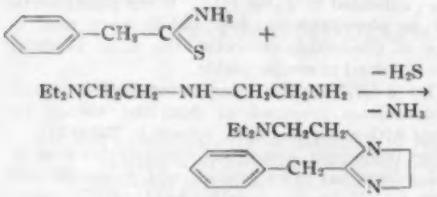
* Received May 5, 1955, from the School of Pharmacy, University of Maryland, Baltimore.
Presented to the Scientific Section, A. PH. A., Miami Beach meeting, May 1955.

Bis-quaternization is, of course, also possible in compounds of this type, although difficulties have been encountered in attempts to quaternize imidazolines unsubstituted in the 1-position (2,5).

In procedures reported for preparation of homogeneous samples of N,N-disubstituted-dialkylenetriamines, only small yields were indicated. N,N-diethyl-diethylenetriamine has been prepared in low yield by reaction of diethylamine, ammonia, and ethylene dibromide (6); and, in yields up to twenty-one per cent, by reaction of diethylamine with ethylenimine under various conditions (7, 8). Low yields of several other N,N-disubstituted-diethylenetriamines were also reported (8). Methods based on procedures used for preparation of N-substituted-ethylenediamines (9, 10) proved useful for preparation of N,N-disubstituted-diethylenetriamines in good yields by reaction of N-(2-chloroalkyl)-dialkyl-ammonium chlorides, 1-(2-chloroethyl)-piperidinium chloride, or 4-(2-chloroethyl)-morpholinium chloride with ethylenediamine either in aqueous or absolute ethanolic media (Eq. 1).

$$R_2\overset{+}{N}(H)CH_2CH(R')Cl^- + H_2NCH_2CH_2NH_2 \xrightarrow{-HCl} R_2NCH_2CH(R')—NH—CH_2CH_2NH_2 \quad (1)$$

1-(2-Diethylaminoethyl)-2-benzyl-2-imidazoline was prepared by procedures described for preparation of 2-benzyl-2-imidazoline from phenylthioacetamide (11), phenylacetic acid (12), or phenylthioacetomorpholide by reaction with N,N-diethyl-diethylenetriamine (Eq. 2).



$$Et_2NCH_2CH_2—NH—CH_2CH_2NH_2 \xrightarrow[-NH_3]{-H_2S}$$

(2)

A series of 1-(disubstituted-aminoalkyl)-2-benzyl-2-imidazolines was prepared from other N,N-dialkyl-dialkylenetriamines by their reaction with phenylthioacetamide. In addition to the usual analytical procedures, the characteristic absorption in the neighborhood of 230 mμ ascribed to the —C=N— linkage of the imidazoline nucleus serves to confirm the structure of the products (13). The preparation of quaternary salts of the imidazolines is in progress and will be reported later together with pharmacologic data.

EXPERIMENTAL

The melting points reported in the following were taken using a Fisher-Johns apparatus.

Substituted-aminoalcohols.—Obtained commercially or prepared by aminolysis of ethylene chlorohydrin or addition of the appropriate secondary amine to ethylene oxide.

2-Di-*n*-propylaminoethanol was prepared by reaction of di-*n*-propylamine with ethylene oxide in 95% ethanol using a procedure which has been applied in the preparation of 2-diethylaminoethanol (14). The product was obtained in 67% yield; b_{26} 95–98°, n_D^{20} 1.4392, d_4^{20} 0.8570; reported values: 195–196°/758 mm., 1.4402, 0.8576, respectively (15).

2-Diisopropylaminoethanol was prepared from diisopropylamine and ethylene oxide by the above method; but better yields were obtained by reaction of diisopropylamine with ethylene chlorohydrin using a method previously reported (16).

1-Piperidine-ethanol was prepared from piperidine and ethylene chlorohydrin (17).

N-(Chloroalkyl)-dialkylammonium Chlorides.—Prepared from the substituted-aminoalcohols by treatment with thionyl chloride in dry chloroform as described for the preparation of 4-(2-chloroethyl)-morpholinium chloride (18).

N,N-Dialkyl-dialkylenetriamines (Tables I and II).—(Method I). A solution of 0.2 mole of the appropriate N-(chloroalkyl)-dialkylammonium chloride in 40 ml. of water was added to a solution of 60 Gm. (1.0 mole) of ethylenediamine in 180 ml. of water; and the mixture was refluxed gently for ten hours. It was cooled, saturated with sodium hydroxide, and extracted with ether. The ether

TABLE I.—N,N-DIALKYL-DIALKYLENETRIAMINES

$$R_2NCH_2CH(R')—N(H)—CH_2CH_2NH_2$$

R_2N	R′	Yield,[a] %	b. p. °/mm.	n_D^{20}	d_4^{20}	Tripicrate m. p.
Dimethylamino-	H	30	115–120/55	1.4550	0.8990	155–157
Dimethylamino-	CH_3	80	115–120/45	1.4603	0.9067	151–152
Diethylamino-	H	65, 50[b]	125–130/40	1.4600	0.8743	200.5–203.0
		20.1 (8)	115–120/25 (8)	1.4629 (7)	0.8761 (7)	204.5–205.5 (7)
Di-*n*-propylamino-	H	9, 87[b]	75–80/0.6	1.4558	0.8551	208.0–209.5
Diisopropylamino-	H	75[b]	83–87/0.8	1.4585	0.8641	198–199
Di-*n*-butylamino-	H	79[b]	99–102/1.0	1.4565	0.8522	202–203
1-Piperidino-	H	68	86–90/0.8	1.4871	0.9325	205–206
		"some" (8)	104–105/4 (8)	1.4881 (8)	0.9378 (8)	204–205 (8)
4-Morpholino-	H	38	165–170/55	1.4877	1.0159	235
		"little" (8)	120–122/4.5 (8)			235–236 (8)

[a] Method I. [b] Method II.

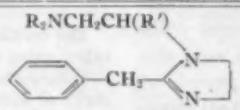
TABLE II.—N,N-DIALKYL-DIALKYLENETRIAMINES (ANALYTICAL DATA)

$R_2NCH_2CH(R')-N(H)-CH_2CH_2NH_2$

R_2N	R′	Formula (free base)	Anal. % N Calcd.	Anal. % N Found	Formula (Tripicrate)	Anal. % N Calcd.	Anal. % N Found
Dimethylamino-	H	$C_6H_{17}N_3.H_2O$	28.16	28.59	$C_{24}H_{26}N_{12}O_{21}$	20.54	20.06
Dimethylamino-	CH_3	$C_7H_{19}N_3$	28.93	28.46	$C_{25}H_{28}N_{12}O_{21}$[a]	20.19	20.16
Di-*n*-propylamino-	H	$C_{10}H_{25}N_3$	22.43	22.53	$C_{28}H_{34}N_{12}O_{21}$	19.22	19.15
Diisopropylamino-	H	$C_{10}H_{25}N_3$	22.43	22.34	$C_{28}H_{34}N_{12}O_{21}$[a]	19.22	19.02
Di-*n*-butylamino-	H	$C_{12}H_{29}N_3$	19.51	19.62	$C_{30}H_{38}N_{12}O_{21}$	18.62	18.63

[a] Picric acid content determined by titration with standard methylene blue solution (19) through courtesy of Myron S. Weinberg. The observed values were 100 ± 1% of those calculated for the tripicrates.

TABLE III.—1-(N,N-DISUBSTITUTED-AMINOALKYL)-2-BENZYL-2-IMIDAZOLINES



R_2N	R′	b. p. °/mm.	Formula (free base)	Anal. % N Calcd.	Anal. % N Found	Dipicrate m. p.	Formula[a] (dipicrate)	Anal. % Calcd.	Anal. % Found
Dimethylamino-	H	145–150/1.5	$C_{14}H_{21}N_3$	18.10	17.90	187–188	$C_{26}H_{27}N_9O_{14}$	18.28	18.22
Dimethylamino-	CH_3	180–184/3.5	$C_{15}H_{23}N_3$	17.12	17.21	217	$C_{27}H_{29}N_9O_{14}$	17.92	18.06
Diethylamino-	H	175–180/4.0	$C_{16}H_{25}N_3$	16.20	16.10	156–158	$C_{28}H_{31}N_9O_{14}$	17.57	17.50
Di-*n*-propylamino-	H	166–171/1.2	$C_{18}H_{29}N_3$	14.62	14.68	159–160	$C_{30}H_{35}N_9O_{14}$	16.90	17.01
Diisopropylamino-	H	167–172/1 3	$C_{18}H_{29}N_3$	14.62	14.60	185–187	$C_{30}H_{35}N_9O_{14}$	16.90	16.67
Di-*n*-butylamino-	H	167–172/0.1	$C_{20}H_{33}N_3$	13.32	13.42	135–136	$C_{32}H_{39}N_9O_{14}$	16.34	16.37
1-Piperidino-	H	177–180/0.6	$C_{17}H_{25}N_3$	15.48	15.48	154–155	$C_{29}H_{31}N_9O_{14}$	17.27	17.20
4-Morpholino-	H	200–205/1.5	$C_{16}H_{23}N_3O$	15.37	15.30	161–161.5	$C_{28}H_{29}N_9O_{15}$	17.23	17.05

[a] Picric acid content determined by titration with standard methylene blue solution (19) through courtesy of Myron S. Weinberg. The observed values were 100 ± 1% of those calculated for the dipicrates.

solution was dried using anhydrous potassium carbonate; and, after distillation of the ether, the product was distilled under reduced pressure.

(Method II). A solution of 0.1 mole of the appropriate N-(chloroalkyl)-dialkylammonium chloride in 30 ml. of absolute ethanol was added slowly to a solution of 30 Gm. (0.5 mole) of ethylenediamine (98–100 per cent) in 60 ml. of absolute ethanol. The mixture was refluxed for five hours. The ethanol and excess ethylenediamine were removed by distillation under reduced pressure, and the residue separated into two layers. The top layer was separated and distilled under reduced pressure.

The product obtained using either of the above methods was further purified by redistillation through a fractionating column until a practically colorless reasonably homogeneous material with a boiling range of not more than 4 to 5 degrees was obtained.

1-(N,N-Disubstituted-aminoalkyl)-2-benzyl-2-imidazolines (Table III).—A mixture of equimolecular amounts of the N,N-dialkyl-dialkylenetriamine and phenylthioacetamide (20) was heated on a steam bath under reduced pressure until evolution of hydrogen sulfide and ammonia ceased. The mixture was distilled under reduced pressure, and the product which was collected after any unreacted N,N-dialkyl-dialkylenetriamine had distilled, was redistilled as necessary to produce a yellow oil (boiling range indicated in Table III). When phenylacetic acid or phenylthioacetomorpholide were used in place of phenylthioacetamide, the same products were obtained in similar yields.

The 1-(N,N-disubstituted-aminoalkyl)-2-benzyl-2-imidazolines prepared as described above, together with analytical data, appear in Table III.

The ultraviolet absorption characteristics of 2-imidazolines has been studied and molar absorptivity ($\epsilon_{max.}$) in the neighborhood of 230 mμ is characteristic of the —C=N— linkage in the 2-imidazolines (13). Spectroscopic data were obtained with solutions of the 1-(N,N-disubstituted-aminoalkyl)-2-benzyl-2-imidazolines in 95% ethanol contained in 1-cm. cells using a Model DU Beckman spectrophotometer. In certain cases, measurements were made before and after acidification with concentrated hydrochloric acid. These data are summarized in Table IV, and are consistent with the structures of the compounds as postulated.

SUMMARY

1. Methods for the preparation of N,N-disubstituted-dialkylenetriamines in satisfactory yields are described, and a series of such compounds has been prepared by those methods.

TABLE IV.—1-(DISUBSTITUTED-AMINOALKYL)-2-BENZYL-2-IMIDAZOLINES ULTRAVIOLET SPECTRAL DATA

2-Benzyl-2-imidazolines	Concentration (moles per liter)	Maximum Absorption	Transmittance, %	Max.
1-(Dimethylaminoethyl)-	1.039×10^{-4}	229	25.3	5,790
1-(1-Dimethylamino-2-propyl)-	1.280×10^{-4}	238	24.1	4,830
1-(Diethylaminoethyl)-	1.114×10^{-4}	230	16.0	7,210
1-(Di-*n*-propylaminoethyl)-	1.287×10^{-5}	238	82.8	6,360
1-(Di-*n*-propylaminoethyl)- (acidified)	1.175×10^{-5}	242	76.3	9,950
1-(Diisopropylaminoethyl)-	1.205×10^{-5}	238	86.0	6,410
1-(Diisopropylaminoethyl)- (acidified)	6.297×10^{-5}	238	19.1	11,430
1-(Di-*n*-butylaminoethyl)-	1.508×10^{-5}	238	80.9	6,160
1-(1-Piperidinoethyl)-	9.396×10^{-5}	238	36.4	4,640
1-(4-Morpholinoethyl)-	9.731×10^{-5}	236	38.8	4,240

2. A series of 1-(disubstituted-aminoalkyl)-2-benzyl-2-imidazolines has been prepared from the N,N-disubstituted-dialkylenetriamines by procedures used for the preparation of 2-imidazolines from ethylenediamine. These compounds and their quaternary salts are of interest pharmacologically because of their structural relationships to 2-benzyl-2-imidazoline and acetylcholine.

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A Fluorometric Assay of Reserpine*

By EARL B. DECHENE

The fluorescence of solutions of reserpine under ultraviolet light is increased by heating with hydrogen peroxide, and is quantitatively related to its concentration. An assay procedure has been developed for tablet preparations containing reserpine alkaloid, and the results of the application of this method to a powdered extract of *Rauwolfia serpentina* is reported.

RESERPINE is now established as the primary active alkaloid of *Rauwolfia serpentina* (1, 2). Analytical methods for its determination have been based on the ultraviolet absorption characteristics of the pure crystalline alkaloid (3, 4). Sakal and Merrill (5) have used this method in conjunction with paper ionophoresis for the determination of reserpine in tablets made with reserpine or with mixtures of Rauwolfia alkaloids.

Banes (6) has reported a color reaction for the determination of reserpine, and McMullen, *et al.* (7), have used the fluorescence shown by solutions of reserpine heated with mineral acids as a method for its quantitative determination.

Reserpine and other Rauwolfia alkaloids fluoresce under ultraviolet light, and this property has been used to locate them on chromatograms (5, 8, 9).

We have found that the intensity of the fluorescence of solutions of reserpine heated with hydrogen peroxide is increased about tenfold over that of solutions not so treated. The intensity of the

* Received April 29, 1955, from the Research & Control Laboratories of Charles E. Frosst & Co., Montreal, P. Q., Canada.

fluorescence was found to be proportional to the concentration of reserpine in solution, and concentrations as low as 0.2 μg. per 10 ml. of solution were measurable.

A fluorometric method was therefore developed to determine reserpine in tablets containing rutin, ascorbic acid, and theophylline. In these assays purification and removal of interfering substances was accomplished by the ability of reserpine to be extracted quantitatively by chloroform from acid or alkaline aqueous solutions (4, 7).

An extraction procedure based on this principle was used also to determine the reserpine content of a powdered extract of *Rauwolfia serpentina* by the fluorometric assay. The values found for the reserpine content by this method, showed close agreement with those obtained using a chromatographic procedure described by Klohs, *et al.* (8), to separate reserpine from the drug, which we combined with the spectrophotometric measurement for its determination.

Though it was not established that the extraction procedure separated reserpine completely from other alkaloids present in the drug, which could fluoresce in the same manner as reserpine, it would appear that the accuracy of the fluorometric method is equal to that of this combined chromatographic-spectrophotometric procedure for the determination of reserpine.

It was established that the intensity of fluorescence shown by reserpine is twenty-eight times as great as that shown by an equal weight of a material containing the "water-soluble" alkaloids of *Rauwolfia serpentina*.

EXPERIMENTAL

Reagents.—Reserpine alkaloid; 5 *N* acetic acid; chloroform; 3% hydrogen peroxide; 1% w/v sodium bicarbonate; 0.01 *N* hydrochloric acid; methanol; quinine sulfate solution containing 0.25 μg. per ml. in 0.1 *N* sulfuric acid.

Apparatus.—Coleman fluorometer, model 12B fitted with PC_1, and B_1 filters; cuvettes; 50-ml. glass stoppered centrifuge tubes.

Effect of Heating with Hydrogen Peroxide on Fluorescence of Reserpine.—Solutions of reserpine alkaloid containing 5 μg. in 100 ml. of 5 *N* acetic acid showed a considerable increase in fluorescence on standing overnight at room temperature in open cuvettes. This change was attributed to oxidation of reserpine by air, since little or no change occurred in the solutions when stored in stoppered tubes under the same conditions. It was found that fluorescence of maximal intensity could be obtained by adding 1.0 ml. of 3% hydrogen peroxide to the solutions, and heating the tubes in a boiling water bath for forty-five minutes. The fluorescence developed in this manner showed no change on standing sixteen hours in open cuvettes at room temperature. The results of these experiments are summarized in Table I.

TABLE I.—FLUORESCENCE OF RESERPINE IN 5 *N* ACETIC ACID SOLUTION

Series[a]	Reserpine, μg./10 ml.	Galvanometer Readings, Time in Hours: 0	1	4	16
A	10	22	34	45	Off scale
	10	22	32	47	Off scale
	20	41	61	81	Off scale
	20	41	61	81	Off scale
		1/4	1/2	3/4	1[b]
B	0.4	11	13	16	16
	0.4	11	14	17	17
	0.8	22	39	34	34
	0.8	22	40	34	34

[a] Series A: in open cuvettes at room temperature. Series B: addition of 1.0 ml. of 3% H_2O_2 and heating in boiling water bath.

[b] The values found for the tubes heated for 1 hour, remained unchanged on standing 16 hours at room temperature.

Relationship between Fluorescence and Concentration of Reserpine.—A solution of reserpine in 5 *N* acetic acid was prepared to contain 0.2 μg. per ml. Duplicate aliquots of this solution were placed in cuvettes to give a range of concentrations from 0.2 to 1.8 μg. of reserpine. To each tube, 1.0 ml. of 3% hydrogen peroxide was added and sufficient 5 *N* acetic acid to bring the volume to 10.0 ml. The tubes were placed in a boiling water bath for forty-five minutes and then cooled to room temperature. The fluorescence was measured in a Coleman fluorometer fitted with Coleman PC_1 and B_1 filters. The instrument was adjusted to give a galvanometer reading of 80 for the fluorescence of a solution of quinine sulfate in 0.1 *N* sulfuric acid containing 0.25 μg. per ml. A plot of the galvanometer readings against the concentration of reserpine in the solutions was made. The slope of the line multiplied by the average of the readings gave reserpine values that agreed satisfactorily with theoretical amounts in the solutions, except at concentrations of 0.2 μg. The results are shown in Table II.

TABLE II

Galvanometer Readings	Av.	Theory μg. of Reserpine	μg. of Reserpine from Curve	% Deviation
8.0, 7.5	7.75	0.2	0.186	−7.0
16, 17	16.50	0.4	0.396	−1.0
34, 34	34.0	0.8	0.816	+2.0
43, 43	43.0	1.0	1.032	+3.2
62, 62	62.0	1.5	1.488	−0.8
75, 75	75.0	1.8	1.8	0

Procedure for the Measurement of Fluorescence.—A standard curve was not used to determine the concentration of reserpine in the assays. Greater accuracy was achieved by measuring the fluorescence of the unknown and the standard at the same time in order to minimize the effect of instrument variability on the readings. Duplicate aliquots of the solutions of the unknown containing an estimated 1.0 μg. of reserpine are placed in cuvettes

containing 1.0 ml. of 3% hydrogen peroxide. The volume in each tube is brought to 10.0 ml. with 5 *N* acetic acid and the solutions are heated for forty-five minutes in a boiling water bath. A solution of reserpine in 5 *N* acetic acid is made to contain 1.0 μg. in 5.0 ml. Duplicate 5.0-ml. aliquots of this solution are used for the standard and are treated in the same manner as the unknowns. The tubes are cooled to 25° and the fluorescence determined as described above.

The concentration of reserpine in the unknown solutions is calculated as follows:

$$\frac{\text{Galvanometer reading of unknown}}{\text{Galvanometer reading of standard}} \times$$

$$\mu\text{g. of resperine in standard solution} = \mu\text{g. of resperine in unknown solution}$$

The Determination of Reserpine in Powdered Extract of *Rauwolfia serpentina*.—A powdered extract of Rauwolfia labeled to contain 7% total alkaloids, was assayed for reserpine as follows: A sample of 3.0 Gm. was mechanically shaken for three hours in an Erlenmeyer flask containing 70 ml. of methanol. The solution was filtered and the funnel and flask washed with additional methanol. The residue, after evaporation to dryness in an evaporating dish, was macerated with 10 ml. of 1 *N* sulfuric acid and transferred to a separatory funnel by means of about 20 ml. of water. The solution was extracted with 6 × 25 ml. of chloroform or until the last extraction gave a negative test for alkaloid with Mayer's reagent. The combined chloroform extracts were washed with 50 ml. of 1% sodium bicarbonate solution, and evaporated to dryness. The residue was shaken with 50 ml. 5 *N* acetic acid, transferred to a 100-ml. volumetric flask and made to volume with 5 *N* acetic acid. On the basis that the powdered extract contained 0.7% reserpine, the solution was diluted with 5 *N* acetic acid to contain 2 mg. reserpine in 100 ml. The ultraviolet absorption of this solution was examined in a Beckman spectrophotometer. A correct maximum for reserpine was shown at 268 mμ, but the absorbance gave values of over 2% for the reserpine content of the material. Further dilutions with 5 *N* acetic acid were made until the solution contained an estimated concentration of 1.0 μg. of reserpine per ml. and the fluorescence determined as described under "Procedure." The results of two assays by this method showed 0.492 and 0.518% for the reserpine content of the powdered extract.

Confirmation of this value was obtained by using the method described by Klohs, *et al.* (8), for the isolation of reserpine from Rauwolfia. A sample of 3.0 Gm. was extracted with methanol as described. The material obtained after removal of the methanol, was macerated with 10 ml. portions of water and centrifuged. The water after the fourth maceration was colorless and all these water extracts were pooled and used to obtain the water-soluble alkaloids of Rauwolfia.

The water-insoluble material was stored in vacuum over concentrated sulfuric acid until dry. It was then macerated with chloroform containing 5% v/v methanol and poured on a column 20 x 40 mm. of 25 Gm. of a 3:1 mixture of 200-mesh silica gel and Celite® wet with the same solvent. The chromatogram was developed with the chloroform-methanol solvent until the lowest colored band was within 5 mm. of the bottom. The absorbent was extruded, allowed to dry slightly in the air, and spotted with Frodhe's reagent. The lower half of the column showed a faint blue fluorescence under ultraviolet light and blue spots with Frodhe's reagent. The section immediately above showed no fluorescence or spots with the reagent. The top portion of the column was dark brown in color and gave an intense greenish fluorescence under ultraviolet light. These three sections were macerated separately in methanol, filtered and washed with methanol until the filtrates were colorless. The solutions were diluted with methanol until absorbances suitable for a Beckman DU spectrophotometer were obtained. Only those solutions made from the lower half of the column, showed absorption curves with a maximum and minimum occurring at wavelengths corresponding to those of pure reserpine (5).

The values found by this method were 0.520 and 0.516% reserpine for two assays of the powdered extract, and are in good agreement with the fluorometric results.

The remaining alkaloids of Rauwolfia were obtained by making the aqueous solution, obtained from the macerations, alkaline with 2 *N* ammonium hydroxide and extracting with 25-ml. portions of chloroform until the last extraction gave no reaction with Mayer's reagent. The combined chloroform extracts were evaporated to dryness and weighed. The fluorescence of a sample of this material was measured as described in the "Procedure," and found to be one twenty-eighth that shown by an equal weight of reserpine.

Method of Assay for Tablets.—The fluorometric method was used to determine reserpine in the tablets containing reserpine alone and in combination with rutin, ascorbic acid, and theophylline.

The average weight per tablet is obtained from the weight of 20 tablets, and 10 tablets are reduced to a fine powder. A weight of the powder equal to 1.0 mg. of reserpine is placed in a 50-ml. stoppered centrifuge tube containing 10 ml. of 5 *N* acetic acid and warmed to 50°. The tube is shaken for three minutes, cooled, and shaken for three minutes with 10.0 ml. of chloroform. After centrifuging, the aqueous layer is drawn off, and the chloroform filtered. The filtrate is placed in a 50-ml. stoppered centrifuge tube containing 10 ml. of 1% aqueous sodium bicarbonate, and shaken for three minutes. After centrifuging, the aqueous layer is drawn off, and 1.0 ml. of the chloroform placed in a 100-ml. volumetric flask containing 5 ml. of 5 *N* acetic acid. The flask is heated on a steam bath to remove the chloroform, and 5 *N* acetic acid added to bring to volume. The fluorescence of duplicate 1.0-ml. aliquots of this solution is determined as described in the "Procedure."

The results of over 100 assays on both laboratory and commercially prepared tablets of the type described gave values for reserpine ranging from 94 to 102% of the label claim.

The recovery of reserpine from known solutions using the same procedure described for the tablets ranged from 98 to 102%.

SUMMARY

The fluorescence under ultraviolet light shown by solutions of reserpine heated with hydrogen peroxide has been used for its quantitative determination in tablet preparations.

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The Determination of Reserpine by Chromatographic Separation from Pharmaceutical Products*

By WILLIAM F. BARTELT and EUGENE E. HAMLOW

A method for the routine determination of reserpine in pharmaceutical preparations is reported. A chromatographic technique is employed to isolate reserpine from complex vehicles and from its decomposition products. The method is simple and accurate and allows ultraviolet measurement of the isolated drug.

DURING the last three years reserpine has become an important therapeutic agent in the treatment of hypertension and other maladies. Its inclusion in a variety of dosage forms has presented several problems to the control chemist such as: (*a*) the quantitative separation of reserpine from its decomposition products and from the diluents and complex vehicles of the dosage forms, (*b*) the isolation of quantities large enough to determine, and (*c*) the determination of the alkaloid after it has been isolated.

The ultraviolet absorption properties of pure reserpine have been reported as a means of determination (1, 2). Reserpine may also be determined colorimetrically by the formation of a chloroform-soluble dye complex (3) and when interfering substances can be eliminated, by reaction with vanillin reagent (4).

When attempting to assay elixirs of reserpine, the problem of separating a relatively small amount of the drug from a large volume of complex vehicle is encountered. Precipitation of the reserpine has been reported (5), but has not been applicable to all vehicles. The dye method (3) is satisfactory when an additional procedure is used to show any possible reserpine decomposition. This paper describes a new chromatographic technique to isolate reserpine from pharmaceutical preparations. The hydrolysis products of reserpine, if present, are eliminated and the determination is completed by ultraviolet measurements.

Routine investigations with paper strip chromatography had shown that reserpine adsorbed to cellulose readily at any *p*H above 2.0. Water appeared to have no elution effect on the adsorbed drug. This indicated a possible method for separating reserpine from the flavors, dyes, and other water-soluble constituents of elixir preparations, as well as from reserpic acid and trimethoxybenzoic acid. Ultraviolet assay for pure reserpine would then be possible.

To accommodate a larger volume of sample than can be efficiently applied to paper strips, an adsorption column of Solka-®floc[1] and Celite®[2] 545 (1:1) was eventually used. Celite® 545 improved the operating characteristics and flow rate of the column.

Reserpine, applied to the top of the column, exhibited no migration when the column was washed with water. Limited amounts of diluted alcohol (27%) caused only a slight migration of reserpine on the column. The vehicle constituents and reserpine hydrolysis products were completely eluted by this technique.

Five normal acetic acid as used by Sakal and Merrill (2) in ionophoresis work, proved to be an

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[1] The Brown Company, Berlin, N. H.
[2] The Johns-Manville Company, N. Y.

excellent eluting solvent for the isolated reserpine and allowed the ultraviolet absorption to be determined directly on the effluent from the column.

The next problem was to ascertain exactly when the reserpine-bearing acetic acid was leaving the column. This would avoid collecting the aqueous hold-over volume of the column, which would dilute the eluted reserpine. The acid concentration of the eluted sample and the matching reagent blank must be equal for an accurate ultraviolet determination. Indication of the acetic acid flow from the column was accomplished by sealing two platinum wires into the wall of the chromatographic tube just above the outlet stem. The wires were connected in series with a 6-volt dry-cell battery and a voltmeter. When only deionized water was in the electrode area, the indicated voltage would be about zero. However, with 5*N* acetic acid in the electrode area the indicated voltage was considerably more, and the meter would indicate 3 to 4 volts. By observing the behavior of the voltmeter, it was possible to determine exactly when to begin collecting the reserpine fraction. The indicated voltage was also used to establish the presence and absence of acetic acid in the column when washing the column in preparation for re-use.

METHOD

Reagents.—(*a*) 5 *N* acetic acid. (*b*) 3% acetic acid-methanol (9:1). (*c*) 27% alcohol. (*d*) 50% alcohol. (*e*) Solka®-floc. (*f*) Celite® 545.

Preparation of the Adsorption Columns.—The chromatographic tubes (2 x 20 cm.) are necked down and joined to a 1-mm. capillary stem about 7 cm. long. Two platinum wires about 1.5 cm. long are sealed into the tube wall about 1 cm. from the stem. The electrodes are connected in series with a 6-volt dry cell and a voltmeter having a 5-volt scale.

Prepare the adsorption columns by slurrying equal weights (about 5 Gm. each) of Solka®-floc and Celite® 545 with water. Transfer the slurry to the tube previously fitted with a glass wool plug and allow to settle, without packing, to a depth of at least 80 mm. Place a second glass wool plug above the column and cover with 0.5 cm. of glass beads. Wash the column with 50 ml. of 5 *N* acetic acid followed by 50 ml. of 50% alcohol. Pass water through the column in 15-ml. portions until the voltmeter reads constant at about 0.2 volt or less. Allow each portion to drain completely into the column before adding additional volumes.

Procedure.—(*a*) *Preparation of the Sample.*—The common liquid preparations may be assayed without prior treatment. If necessary, the *p*H of the product should be appropriately adjusted to *p*H 2.5-6.0 and the alcohol content to below 30%.

Grind tablets and weigh tablet mixture equivalent to 2.5 mg. of reserpine. Transfer the mixture to a 50-ml. volumetric flask calibrated to contain the tablet mixture plus 50 ml. of solvent. Add 30 ml. of the acetic acid-methanol solvent to the flask and heat on a steam bath for fifteen minutes with occasional shaking. Cool the solution, bring to volume, and mix well. Centrifuge a 15-ml. portion and remove exactly 10 ml., representing 0.5 mg. of reserpine, for assay.

(*b*) *Separation of Reserpine.*—Allow the column head to drain completely. Introduce into the column a volume of solution containing 0.5 mg. of reserpine, and allow it to pass into the column. Add 100 ml. of water in about 15-ml. portions, allowing the final portion to drain completely into the column. Wash the column with 30 ml. of 27% alcohol followed by 50 ml. of water to remove the alcohol from the column. Allow the column to drain.

Elute the adsorbed reserpine from the column with 60 ml. of 5 *N* acetic acid, added in small portions to maintain constant flow through the column. Collect a 50-ml. fraction when the acetic acid has entered the electrode area, as shown by a rise in indicated voltage on the voltmeter. When the acetic acid has passed into the column, add water to purge enough acetic acid from the column to make exactly 50 ml.

The column may be prepared for re-use by washing with water until the indicated voltage drops to a minimum.

(*c*) *Determination of the Reserpine.*—Mix the acetic acid eluate well and determine the absorbance against a 5 *N* acetic acid blank, at 275, 267, and 259.25 mμ. The absorbances at 275 mμ and at 259.25 mμ are the same for pure reserpine. The ratio A_e 267/A_e 275 is indicative of sample purity and should be about 1.141, but may vary with the instruments used. Calculate total reserpine from the absorbance at 267 mμ. The absorbance index (a_s) for reserpine is 27.8.

DISCUSSION

The 100 ml. of water used to wash the column is sufficient to clear the column of viscous elixir vehicles. Simpler vehicles and solvents may not require this amount. The subsequent 27% alcohol wash was found necessary to remove specific vehicle constitutents not removed by the aqueous wash which eluted with the acetic acid and contaminated the reserpine fraction. The concentration and volume of alcohol are limited because this step causes slow migration of the reserpine.

The wavelengths at which the solutions are read are those indicated by Sakal and Merrill (2). The ultraviolet characteristics of pure reserpine should be investigated on the specific instrument to be used. Occasionally a sample may exhibit contamination. If the contamination cannot be eliminated, the method of Sakal and Merrill (2) may prove useful in correcting for the contaminant.

Reproducibility.—The standard error per single assay, estimated from replicate assays on 18 samples, was S = 1.20 per cent with 24 degrees of freedom. This gave a 95% confidence limit of ±2.5%, meaning that, on the average, 95% of the assays will be within 2.5% of true value.

Behavior of Reserpine Degradation Products.—Reserpine has been shown to be liable to hydrolysis in certain aqueous vehicles and at elevated storage

temperatures, yielding reserpic acid and 3,4,5-trimethoxybenzoic acid. These products of hydrolysis, unless eliminated by the assay procedure, exhibit ultraviolet absorption leading to an inaccurate determination of intact reserpine present.

Reserpic acid, applied to the column in 2% citric acid-alcohol solution (6:1), was carried through in the water wash, and none was found in the 5 *N* acetic acid eluate from the columns. Assayed reserpine elixir, to which had been added reserpic acid equivalent to 25% of the reserpine present, showed no greater amount of reserpine present than the elixir without reserpic acid.

Equivalent amounts of reserpic acid and trimethoxybenzoic acid, added in citric acid-alcohol solution, were returned by the column in the water wash. Nothing was found in the acetic acid eluate. Similarly, reserpine elixir with reserpic acid and trimethoxybenzoic acid added showed the same assay as the elixir without the added hydrolysis products.

Operational Characteristics of the Columns.—The normal flow rate of the column is 30–40 drops per minute. Slower flow-rates, found when columns were packed more tightly, did not increase the efficiency of the operation. The loosely packed column, formed by normal settling of the bed, has a faster flow-rate which shortens the time required for an assay. The moderate channeling which occurs has never caused difficulties.

When using a 6-volt battery, the voltmeter indicates 0 to about 0.2 volt when the vehicles or solvents have been washed from the column after adsorption of the reserpine. Collection of the acetic acid eluate is begun when not more than 1 volt is indicated on the meter. Some eluted reserpine may be lost if time is allowed for the voltage to rise above 1 volt, because the R_f value of reserpine with the 5 *N* acetic acid solvent is 1. Approximately 95% of the reserpine is eluted in the first 20 ml. of acetic acid.

To facilitate the assay of a large number of samples a multiple column setup may be constructed. Four duplicate assays can easily be carried out in an eight-hour day, when using three columns. A single assay requires about two and one-half hours, plus time for the preparation of the column and sample.

SUMMARY

1. A chromatographic method for the separation of reserpine from the vehicles and diluents of pharmaceutical preparations has been presented. Hydrolysis products of reserpine are also eliminated in the separation.

2. A technique for recovering the adsorbed reserpine in a solution suitable for ultraviolet measurement of the intact reserpine is reported.

3. The simplicity of the procedure makes it a desirable method for routine analysis of reserpine preparations.

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Effect of Thiamine Hydrochloride on the Stability of Solutions of Crystalline Vitamin B_{12}*

By BEATE A. FELLER and THOMAS J. MACEK

Crystalline vitamin B_{12} is stable in aqueous solutions of thiamine hydrochloride at *p*H 3.5 to 4.5 during prolonged storage at room temperature. Decomposition of vitamin B_{12} occurs at elevated temperatures in the presence of thiamine decomposition products or the thiazole moiety. The amount of vitamin B_{12} decomposed is shown to be related to the stability of the thiamine hydrochloride. Crystalline vitamin B_{12} is stable in solutions of thiamine hydrochloride and niacinamide at *p*H 3.5 to 4.5 and in B vitamin solutions at *p*H 3.0 and 4.5 at normal storage temperatures. Destruction of the vitamin B_{12} in these solutions occurs at elevated temperatures.

PREVIOUS REPORTS (1, 2) indicated that decomposition of crystalline vitamin B_{12} was likely to occur in the presence of thiamine hydrochloride when simple aqueous solutions were heated. However the first paper also emphasized that such solutions could be kept at normal storage temperatures for long periods of time without significant decomposition of vitamin B_{12}. Studies reported here were performed to examine the compatibility of crystalline vitamin B_{12} and thiamine hydrochloride more critically.

The stability of aqueous solutions of thiamine hydrochloride has been reported to be adversely affected by certain buffers, and metals (3, 4). Stability is also affected by *p*H. Decomposition

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products of thiamine therefore could easily be formed in quantities comparable to the concentrations of crystalline vitamin B_{12} often present in aqueous solutions. On this basis it seemed likely that any incompatibility with vitamin B_{12} might be explained in terms of thiamine decomposition products rather than thiamine itself. The effects of decomposed thiamine and combinations of thiamine and niacinamide and other B vitamins on the stability of crystalline vitamin B_{12} were therefore investigated.

EXPERIMENTAL AND RESULTS

The first experiments were designed to study the effect of thiamine hydrochloride on the stability of crystalline vitamin B_{12} at elevated temperatures.

Solutions containing 100 micrograms of crystalline vitamin B_{12} U. S. P. and 50 mg. of thiamine hydrochloride U. S. P. in each cc. were adjusted to *p*H 2 with hydrochloric acid and *p*H 4 with sodium hydroxide. Similar solutions containing only the thiamine hydrochloride were prepared. Samples were heated in an autoclave at 120°, changes in the solution were determined photometrically by measurement of absorption at 5500 Ångströms, using the corresponding thiamine hydrochloride solution as the reference liquid. For this initial test it was found better to measure color at 5500 Å. rather than U. V. absorption at 3610 Å. to reduce interference by thiamine. It is recognized that this would not be an absolute measure of cyanocobalamin (5). Control solutions containing only crystalline vitamin B_{12} were also prepared. These assay data are summarized in Table I.

TABLE I.—STABILITY OF CRYSTALLINE VITAMIN B_{12} AT 120° IN AQUEOUS SOLUTIONS OF THIAMINE HYDROCHLORIDE

	*p*H 2		*p*H 4	
	B_{12} + B_1 HCl	B_{12} Control	B_{12} + B_1 HCl	B_{12} Control
120°/10 min.	− 9%	...	− 4%	...
120°/20 min.	−33%	...	−19%	...
120°/30 min.	−73%	...	−35%	...
120°/60 min.	−80%	−14%	−62%	−3%

The data in Table I show that at 120° rapid change in the vitamin B_{12} content of the solutions occurred in the presence of thiamine hydrochloride. At *p*H 2 the change was greater than at *p*H 4, with or without thiamine hydrochloride.

The effect of varying concentrations of thiamine decomposition products on the stability of crystalline vitamin B_{12} was then investigated.

A stock solution of thiamine hydrochloride U. S. P. containing 20 mg. per cc. was prepared by dissolving the solid in distilled water. Part of the solution was then adjusted to *p*H 8.0 with sodium hydroxide. Progressive decomposition of the thiamine was obtained by autoclaving aliquots of the adjusted solution for various periods of time at 120°. The autoclaved solutions were readjusted to *p*H 4.0 with hydrochloric acid after cooling. They were than assayed for thiamine hydrochloride content by the silicotungstic acid precipitation method described in the British Pharmacopoeia (6).

A solution of crystalline vitamin B_{12} U. S. P. was added to each of the thiamine solutions, including an unheated portion. The solutions were adjusted to *p*H 3.5 with a suitable amount of hydrochloric acid. The solution was adjusted to contain 20 micrograms of crystalline vitamin B_{12} and the equivalent of an original concentration of 10 milligrams of thiamine hydrochloride per cc. The test solutions so prepared contained respectively 0, 2, and 22% decomposed thiamine. Samples of each solution were subdivided into 10-cc. ampuls and flame sealed. Some ampuls were autoclaved at 120°; others were heated at 80° and at 40°. At suitable intervals all were assayed for vitamin B_{12} by the U. S. P. microbiological method (7). Assay data are summarized in Table II.

The data in Table II indicate a progressive loss of vitamin B_{12} at 80 and 120° with increasing amounts of decomposed thiamine hydrochloride. The rate of decomposition was greater at 120°. At *p*H 3.5 decomposition of crystalline vitamin B_{12} was not excessive after storage at 40° for two months even in the presence of decomposed thiamine.

On the basis of these early experiments it seemed logical to determine if thiamine itself or one of its decomposition products was responsible for decomposition of the vitamin B_{12}. The effect of a combination of thiamine hydrochloride and niacinamide on the stability of crystalline vitamin B_{12} was also investigated.

Solutions were therefore prepared containing 2-methyl-4-amino-5-hydroxymethylpyrimidine hydrochloride and of 4-methyl-5-β-hydroxyethylthiazole as representative of the decomposition products of the thiamine molecule (8). The amount of the thiazole and pyrimidine compounds used per cc. was calculated to be equivalent to the amount present in 10 mg. of thiamine hydrochloride.

TABLE II.—STABILITY OF CRYSTALLINE VITAMIN B_{12} AT ELEVATED TEMPERATURES IN AQUEOUS SOLUTIONS OF THIAMINE OF VARYING DECOMPOSITION

	Decomposed B_1 HCl 0%			Decomposed B_1 HCl 2%			Decomposed B_1 HCl 22%		
	*p*H	μg. B_{12} per cc.	% Loss	*p*H	μg. B_{12} per cc.	% Loss	*p*H	μg. B_{12} per cc.	% Loss
Initial	3.53	21.4	..	3.61	21.4	..	3.57	22.0	..
120°/15 min.	3.53	21.4	0	3.56	21.6	0	3.58	16.8	24
120°/45 min.	3.36	17.7	17	3.46	15.0	30	3.50	8.9	60
80°/10 hr.	3.50	21.4	0	3.56	21.4	0	3.52	18.7	15
80°/25 hr.	3.40	20.1	6	3.45	17.1	20	3.40	12.7	41
40°/2 mo.	3.36	20.4	4	3.40	20.4	5	3.31	21.2	4

TABLE III.—COMPOSITION OF TEST SOLUTIONS

	I	II	III	IV	V	VI
Cryst. Vitamin B_{12}	25 μg.	25 μg.	25 μg.	25 μg.	25 μg.	25 μg.
Thiamine HCl	...	10 mg.	10 mg.	...	...	...
Niacinamide	...	...	10 mg.	...	...	...
38% Decomposed Thiamine HCl	...	...	...	6.24 mg.[a]	...	...
Thiazole Moiety	...	...	...	...	4.2 mg.[a]	...
Pyrimidine Moiety	...	...	...	...	...	5.8 mg.[a]
Distilled Water, to	1 cc.	1 cc.	1 cc.	1 cc.	1 cc.	1 cc.

[a] Equivalent to 10 mg. thiamine HCl

TABLE IV.—LOSSES OF VITAMIN B_{12} IN TEST SOLUTIONS AT *p*H 4

	Control I	B_1 only II	B_1 and Niacinamide III	Decomp. B_1 IV	Thiazole V	Pyrimidine VI
120°/15 min.	...	...	44%	...	58%	...
120°/30 min.	0	28%	83%	51%	96%	0
120°/1 hr.	0	63%	...	81%	...	0
40°/3 mo.	...	0	31%	19%	10%	...
40°/4 mo.	3%	18%	35%	28%	19%	3%
RT/6 mo.	No significant loss					

All solutions were adjusted to *p*H 4 with hydrochloric acid but were unbuffered. The composition of the test solutions is given in Table III.

The solutions were subdivided into glass ampuls and flame-sealed. They were assayed for vitamin B_{12} by the U. S. P. microbiological method at suitable intervals during storage at 120°, 40°, and room temperature. Losses of vitamin B_{12} calculated from the assay data are summarized in Table IV.

The data summarized in Table IV indicate that decomposition of crystalline vitamin B_{12} in the presence of thiamine hydrochloride at elevated temperatures is caused by the thiazole and not the pyrimidine. The data also show that at elevated temperatures crystalline vitamin B_{12} is unstable in an aqueous solution containing both thiamine hydrochloride and niacinamide (Solution III) or in a decomposed thiamine solution (Solution IV). At room temperature the decomposition of vitamin B_{12} observed in these solutions was not significant. Assays of the solutions stored at 40° for three months indicated less than 5% loss of thiamine. No greater loss of thiamine was encountered in solutions containing niacinamide.

Another series of solutions was prepared to test the stability of crystalline vitamin B_{12} in aqueous solutions of the B vitamins. Two of these were parenteral solutions, a third was a flavored B vitamin-iron elixir. Their compositions are given in Table V. The parenteral solutions were sterilized by aseptic filtration through microporous porcelain candles; they were subdivided into ampuls and flame-sealed.

Microbiological assay data for vitamin B_{12} in the parenteral solutions stored at room temperature for one year are summarized in Table VI. These parenteral solutions (Solutions A and B) as well as two others of like composition and *p*H containing in addition 1 mg. calcium chloride and 2 mg. magnesium chloride per cc. (Solutions C and D) were subjected to a heating test at 120°. Samples for microbiological assay were taken after 30, 60, and 90 minutes of heating. These assay data are summarized in Table VII.

The elixir was stored at room temperature and at 40° in screw-capped amber bottles. Microbiological assay data during storage for 1 year are summarized in Table VIII.

TABLE V.—COMPOSITION OF B VITAMIN SOLUTIONS

	Solutions mg. per cc.		Mg. per 4 cc.
	Parenteral Solution A	Parenteral Solution B	B Vitamin-Iron Elixir
Cryst. vitamin B_{12}	15 μg.	15 μg.	10 μg.
Thiamine HCl	10.0 mg.	10.0 mg.	2.0 mg.
Riboflavin	0.5	0.5	...
Niacinamide	25.0	25.0	15.0
Calcium pantothenate, dextrorotatory	5.0	5.0	0.64
Pyridoxine HCl	5.0	5.0	1.0
Benzyl alcohol	15.0	15.0	...
Sodium chloride	9.0	9.0	...
Dilute HCl, to adjust to	*p*H 3.0	*p*H 4.5	*p*H 4.2
Glycerin	...	...	0.8 cc.
Ethanol	...	...	0.8 cc.
Syrup (flavored)	...	...	0.8 cc.
Ferrous sulfate	...	...	20.0 mg.
Distilled water	q. s.	q. s.	q. s.

TABLE VI.—STABILITY OF CRYSTALLINE VITAMIN B_{12} IN PARENTERAL SOLUTIONS CONTAINING OTHER B VITAMINS AT R.T.

	μg./cc. (A) pH 3.0	(B) pH 4.5
Initial assay	15.7	16.1
After 3 mo. at room temperature	14.4	17.1
After 6 mo. at room temperature	15.9	17.2
After 1 yr. at room temperature	15.9	14.4

TABLE VII.—STABILITY OF CRYSTALLINE VITAMIN B_{12} IN AQUEOUS SOLUTIONS OF THE B VITAMINS AT 120° C.

Solution	μg./cc. A pH 3.0	B pH 4.5	C[a] pH 3.0	D[a] pH 4.0
Initial assay	15.7	16.1	11.3	12.7
After 30 min.	15.1	14.6	11.6	13.3
After 60 min.	14.9	10.5	10.5	8.2
After 90 min.	6.5	5.62	7.6	2.6

[a] Identical with A and B, but contain calcium chloride and magnesium chloride.

TABLE VIII.—STABILITY OF CRYSTALLINE VITAMIN B_{12} IN A B VITAMIN-IRON ELIXIR

	μg./cc. Room Temperature	40°
Initial assay	11.0	11.0
After 1 mo.	...	10.0
After 3 mo.	9.5	9.5
After 6 mo.	10.4 (5% loss)	9.8 (11% loss)
After 1 yr.	10.6 (4% loss)	5.0 (55% loss)

It is apparent from these data that solutions of the B vitamins can be prepared in which crystalline vitamin B_{12} is stable for long periods of time at normal storage temperatures. On the other hand, large losses of vitamin B_{12} were found after one hour of heating at 120°.

SUMMARY

1. At room temperature crystalline vitamin B_{12} was found to be stable in aqueous solutions at *p*H 4 containing 10 mg. per cc. of thiamine hydrochloride alone or in combination with 10 mg. per cc. of niacinamide.

2. Decomposition of crystalline vitamin B_{12} in aqueous solution was increased appreciably in the presence of thiamine or thiamine decomposition products at elevated temperatures.

3. At elevated temperatures the thiazole moiety of the thiamine molecule decomposes crystalline vitamin B_{12} in aqueous solutions adjusted to *p*H 4.

4. Data are given to show that crystalline vitamin B_{12} is stable in B vitamin solutions at *p*H 3.0 and 4.5 for long periods of time at normal storage temperatures but not when these are heated at 120°.

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A Spectrophotometric Assay for Terpin Hydrate*

By HERBERT PLATT and ARTHUR E. JAMES

A spectrophotometric method of assay involving the treatment of terpin hydrate with phosphomolybdic acid under controlled conditions has been developed. By use of this procedure, the terpin hydrate content of pharmaceutical products may be determined by comparison with a suitable reference standard.

AT PRESENT there is no official assay for terpin hydrate alone, or for it in any of its preparations. The A. O. A. C. (1) developed a complex gravimetric procedure for the determination of terpin hydrate in elixirs. This involves extraction with 10% sulfuric acid followed by extraction with petroleum benzin and finally with a mixture of chloroform and alcohol. Since there is no comparatively simple assay for terpin hydrate, it seemed pertinent that an effort be be made to develop one.

Several color reactions involving terpin hydrate are described in the literature (2–5). Preliminary experimentation indicated phosphomolybdic acid (5, 6) to be the most satisfactory color reaction. The initial blue color produced alters to a stable greenish-blue hue after a fixed time period. While there is some uncertainty as to the exact nature of the reaction involved (7–10) the color is considered to be due to the formation of molybdenum blue, a reduction product of phosphomolybdic acid. Irrespective of the mechanism involved or the nature of the products formed, it was found that the amount of molybdenum blue produced was directly proportional to the concentration of terpin hydrate present.

EXPERIMENTAL DEVELOPMENT OF PROCEDURE

Absorption Maximum.—Five milliliters of elixir terpin hydrate[1] prepared according to the National Formulary (11) was pipetted into a 100-ml. flask and distilled water added to the mark. This served as the standard working solution. The phosphomolybdic acid solution was prepared by acidifying the molybdate-phosphate solution as described in Remington's Practice of Pharmacy (12). To a 100-ml. volumetric flask were added in this order: 5 ml. molybdophosphate solution, 1.50 ml. of the diluted elixir (1.28 mg. terpin hydrate), and 6 ml. concentrated sulfuric acid (specific gravity 1.84) in 2-ml. portions, with agitation of the flask after each addition of the acid.[2] The sample was allowed to cool for fifteen minutes before dilution, thus permitting the maximum intensity of color to develop. The sample was then diluted to the mark with distilled water. An aliquot portion was transferred to one of the matched cuvettes of the spectrophotometer.[3] A blank, containing each of the reagents except the terpin hydrate elixir, was placed in the other cuvette. The absorption spectra of the greenish-blue coloration was measured nine minutes after dilution between 400 and 800 mμ. The reason for the selection of the nine-minute interval is shown later. The data obtained were transferred to graphic form as shown in Fig. 1. This shows absorption maxima

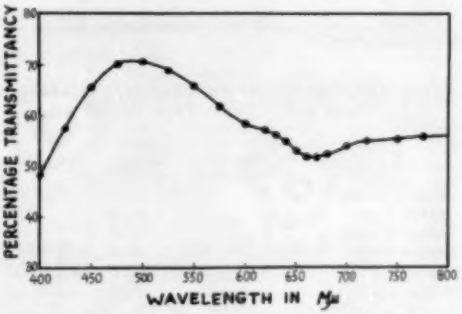

Fig. 1.—Percentage transmittance of terpin hydrate-molybdophosphate solution after an interval of 9 minutes.

at about 400 and at 660 mμ. The latter wave length was selected for use as the spectrophotometer is more sensitive in this area.

Color Stability and Time.—Since the maximum intensity of color developed quickly but soon faded to a shade which seemed to be practically constant, it appeared advisable to make a time study of the color stability. Solutions having terpin hydrate concentrations between 0.85 and 2.55 mg./100 ml. were prepared. The percentage transmittance of each sample was measured at intervals from one and one-half to nine minutes at 660 mμ. The data obtained are shown in Fig. 2. A consideration of these curves indicates that the color reached relative stability in seven and one-half minutes. It was further observed that no significant changes in transmittance were found after the solutions stood for two days. Accordingly, all subsequent measurements were made nine minutes after the reacting substances were mixed.

Beer's Law and Concentration Limits.—Working at 660 mμ the color produced by terpin hydrate and

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[1] Mallinckrodt, U. S. P. grade. Other ingredients in the elixir were found to have no effect on the reduction of the phosphomolybdic acid.

[2] A volume of H_2SO_4 greater than 6 ml. will cause charring, thereby making colorimetric measurements impossible.

[3] A Coleman Universal Spectrophotometer, Model 14, having cuvettes 15 mm. in diameter, was used to measure the transmittance.

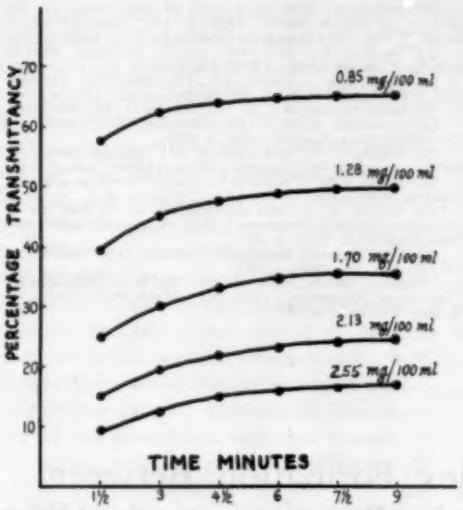

Fig. 2.—Variation of percentage transmittance of terpin hydrate-molybdophosphate solution with time.

molybdophosphate solution as previously described was measured using concentrations ranging from 0.85 to 2.55 mg./100 ml. The data obtained gave a straight line when per cent transmittance was plotted against concentrations on semilog paper indicating adherence to Beer's law within these concentration limits.

APPLICATION OF METHOD TO PROPRIETARY PRODUCTS[4]

The terpin hydrate content of various proprietary products was determined by using the procedure previously described. The content of terpin hydrate was read from the graph prepared to verify adherence to Beer's law. The terpin hydrate content of twenty elixirs is shown in Table I. Each per cent transmittance (%T) listed is the average of three separate measurements.

TABLE II.—COMPARISON OF SPECTROPHOTOMETRIC AND A. O. A. C. METHODS FOR ASSAYING TERPIN HYDRATE ELIXIRS

Sample No.	Terpin Hydrate Found (Gm./100 ml.) Spectrophotometric Method	A. O. A. C. Method
1	1.72	1.72
6	1.76	1.75
8	1.75	1.72

Terpin Hydrate in Specialties

Sample No. 21.—A microcrystalline suspension with menthol and eucalyptol labeled to contain 3.52 Gm. terpin hydrate per 100 ml. gave a value of 3.58 Gm.

Sample No. 22.—Tablets* containing codeine sulfate, tincture of belladonna, ammonium chloride, extract of glycyrrhiza, and fluidextract of ipecac, labeled to contain 0.16 Gm. terpin hydrate gave experimental values averaging 0.15 Gm.

Sample No. 23.—Tablets[5] containing ammonium chloride, extract senega, extract lobelia, powdered ipecac, and antimony and potassium tartrate, labeled to contain 0.13 Gm. terpin hydrate gave an average value of 0.12 Gm.

Sample No. 24.—A liquid preparation containing chloroform, creosote and sodium and potassium glycerophosphates with terpin hydrate could not be assayed by the spectrophotometric method presented herewith, apparently due to the interference caused by the glycerophosphates.

Sample No. 25.—A liquid preparation containing codeine phosphate, prunus virginiana, prunus strobus, and sanguinaria, labeled to contain 0.59 Gm. terpin hydrate in the volume assayed gave a value of 0.62 Gm.

Comparison of Spectrophotometric and A. O. A. C. Methods for Assaying Terpin Hydrate.—A study was made to compare the accuracy and the time involved in assaying terpin hydrate by the spectrophotometric and by the A. O. A. C. methods. After a standard reference curve has been estab-

TABLE I.—TERPIN HYDRATE IN ELIXIRS[3]

Sample No.	% T	Gm./100 ml. Found	% Deviation ±	Sample No.	% T	Gm./100 ml. Found	% Deviation ±
1	34.7	1.72	1.2	11	36.9	1.66	3.4
2	40.3	1.54	9.4	12	34.4	1.72	1.2
3	30.7	1.87	10.0	13	35.5	1.71	0.6
4	30.4	1.88	10.8	14	34.0	1.75	2.9
5	35.2	1.70	0.0	15	35.5	1.71	0.6
6	33.6	1.76	3.5	16	32.6	1.79	5.3
7	32.6	1.79	5.3	17	31.3	1.86	9.4
8	34.1	1.75	2.9	18	35.3	1.70	0.0
9	34.1	1.75	2.9	19	34.3	1.73	2.0
10	35.2	1.70	0.0	20	35.7	1.68	1.1

[3] Samples 1–10 were elixir terpin hydrate labeled to contain 1.70 Gm./100 ml. Samples 11–20 were elixir terpin hydrate with codeine labeled to contain 1.7 Gm./100 ml.

[4] The authors are indebted to the following companies for supplying the products used in this work: Abbott Laboratories, American Pharmaceutical Co., J. W. S. Delavau, Inc., Irwin, Niesler and Co., Eli Lilly and Co., S. E. Massengill Co., Parke, Davis and Co., Sharp and Dohme, Inc., E. R. Squibb and Sons, Upjohn Co., and Henry K. Wampole and Co., Inc.

[5] The tablets were assayed by selecting twenty, grinding them to a fine powder, weighing a suitable portion and transferring it to a volumetric flask. A small volume of distilled water was added and the mixture let stand for an hour with occasional agitation. The mixture was filtered and diluted to volume. Aliquot portions were withdrawn and assayed as previously described.

lished for the spectrophotometric method, an assay for terpin hydrate content can be completed in twenty to twenty-five minutes. In contrast to this it requires approximately two hours to complete the extractions and weighing as described in the A. O. A. C. method (1). The data shown in Table II give a basis for comparing the results obtained by the two methods of assay.

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Study of Possible Complex Formation Between Macromolecules and Certain Pharmaceuticals IX*

Formation of Iodine-Iodide Complexes with Polyethylene Glycol

By DAVID E. GUTTMAN† and TAKERU HIGUCHI

Investigation of the complexing reaction between polyethylene glycol and iodine in an iodine-potassium iodide aqueous system has shown that at high triodide ion concentrations and low free iodine concentrations an insoluble complex having a stoichiometric ratio of one mole of potassium triodide to approximately 10 monomeric units of the polymer is reversibly formed. Potassium ion is apparently a prerequisite for formation of this complex. At much higher free iodine concentrations a second insoluble complex containing a considerable amount of iodine *per se* is reversibly formed from the first. The stoichiometry of the second complex formed appears to be such that one molecule of iodine is taken up by two monomeric units of ethylene oxide in the polymeric chain. The concentration dependencies of both complexing reactions on iodide, potassium ion and free iodine are presented in this report. The interaction in a sodium iodide–iodine system also results in the formation of an insoluble complex. This interaction does not appear to be analagous to that in the potassium iodide–iodine system. Bromine in the presence of bromide ion has also been shown to form a reversible insoluble complex with polyethylene glycol.

THE SIMILARITY in complexing behavior of polyethylene glycols, polypropylene glycols, and polyvinvylpyrrolidone with phenol, substituted phenols, substituted benzoic acids and phenobarbital in aqueous solution (1, 2, 3) indicates a general tendency of water-soluble polyfunctional organic macromolecules to interact with small molecules. It is not surprising, therefore, that the higher molecular weight polyethylene glycols interact with iodine in a manner similar to other water-soluble macromolecules. Although the interaction of polyvinylpyrrolidone (PVP) with iodine has been extensively investigated by Oster and others (4, 5), the analogous reaction of polyethylene glycol (PEG) has received scant attention. The present communication is concerned with the results of a study made on the latter system.

The behavior of PEG toward iodine is markedly similar to that of PVP in certain respects and dissimilar in others. Notably the two systems are alike in that both polymers appear to undergo the complexing reaction only in the presence of iodide ions (4). On the other hand, PVP appears to form highly water-soluble complexes with iodine, whereas, according to the present findings, PEG complexes with iodine are extremely water insoluble. As will be seen, this very lack of water solubility of the PEG com-

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plexes permits a line of experimental study which is not possible with the PVP system.

The existence of solid PEG iodine complexes has previously been indicated by reported pharmaceutical incompatibilities arising from the attempted incorporation of potassium iodide-iodine solutions into PEG ointment bases (6). Although Zimakov and Girshberg (7) postulated a complex to be $O(CH_2CH_2)_2I_2$, no experimental investigation of the interaction has been reported in the available literature.[1] The iodine solubilizing effect of high concentrations of ethylene, diethylene, and triethylene glycols in the absence of iodide ion has been rationalized by Osol and Pines (8) to be due to complex formation of a Lewis acid-base nature. Allawala and Riegelman supported this assumption with their studies on iodine-surface active agent interactions (9). The present study differs from these earlier investigations in that it is concerned with the interaction of much higher molecular weight polyethylene glycols with iodine in the presence of alkali iodides in aqueous solution. Although the nature of the interaction is intrinsically dependent on the cations present, this investigation is concerned chiefly with phase studies on the polyethylene glycol, iodine, potassium iodide aqueous system.

RESULTS AND DISCUSSION

Qualitative Observations.—A certain amount of information as to the nature of the complexing reaction can be gained by visible observation of simple test tube experiments on the system. Thus, when iodine crystals are added to an aqueous solution of PEG (mol. wt. 4000–6000) free from iodide ion, the amount of iodine going into solution appears to be no different than that dissolving in a corresponding amount of distilled water. Quantitative determination of the possible effect of the polymer on the water solubility of iodine is not readily possible because of the formation of traces of colloidal precipitate in the system. This behavior is possibly due to production of a small amount of iodide ions from the free iodine through hydrolysis. If a limited amount of iodine crystals is added to a solution of the polymeric glycol having an approximate molecular weight of 6000 and containing for example, 0.1 mole of potassium iodide per liter, almost instantaneous separation of a well-defined red-brown precipitate occurs. Addition of excess iodine results in transformation of the primary red-brown precipitate to a visibly different, jet-black secondary precipitate.

Over-all Phase Diagram of the System.—Results of quantitative experimental studies of the complexing reaction are summarized partially in Fig. 1. In this figure the concentration of total titratable iodine remaining in solution has been plotted as a function of the millimoles of free iodine crystals added per liter of potassium iodide solution containing varying concentrations of PEG. The concentration of the glycol (Carbowax® 6000) has been expressed in terms of gram monomeric units (or equivalent units) per liter, since, as will be seen later, stoichiometric relationships can be more conveniently calculated on this basis.

$$\text{Concentration of PEG in equivalents per liter} = \frac{\text{concentration of PEG in Gm. per liter}}{44\ (\text{mol. wt. of the ethylene oxide unit})}$$

It is evident from the curves shown in Fig. 1 that at least two complexes of quite different character are formed between the reactants. By following the curve obtained, for example, at an initial PEG concentration of 29.7×10^{-3} equivalents per liter, it can be seen that in the region of the first plateau AB, the equilibrium concentration of total titratable iodine in solution is very low and essentially independent of the iodine concentration initially added to the system, the major portion of the added iodine being precipitated in the form of an insoluble complex. Determination of residual iodide ion concentration established that in this region iodide is precipitated with iodine in an approximate one-to-one ratio as shown in Table I. It was qualitatively established, by determination of residual potassium ion concentration with sodium tetraphenyl boron, that potassium ion precipitation accompanies removal of iodine and iodide from solution in this region.

A previous study has shown that the transient or permanent cloudiness observed when aqueous PEG solutions are treated with phenol is an excellent qualitative test for the presence of this polymer in solution. Application of this simple test to the system under investigation revealed that some polymer remains in solution until point B. Beyond point B, however, the test showed that all of the polymer had been precipitated. The apparent precipitation of 29.7×10^{-3} equivalents of polymer was accompanied by the removal from solution of 3.08×10^{-3} moles of iodine. A stoichiometric ratio of one iodine bound to approximately every ten repeating units of the polymer is thus indicated. It can be seen from the figure that at this point the equilibrium concentration of titratable iodine is no longer invarient but increases abruptly until point C where a second iodine invarient plateau is established. A transition from the primary red-brown precipitate to the secondary black precipitate was visibly apparent along the BC break.

It can be seen from the figure that after apparent complete removal of the polymer from solution, further additions of free iodine to the system resulted in additional take up of the free halogen by the precipitated phase. Determination of residual iodide concentration in this region indicated that this precipitation of iodine did not result in further significant concomitant precipitation of iodide ion. The second iodine invarient plateau is seen to extend to point D, at which point the polymer is apparently saturated with respect to iodine. The

[1] After completion of this manuscript the work of Wurzschmitt [*Z. anal. Chem.*, 130–131, 105(1950)] came to our attention. Although many of the qualitative aspects of this system were observed by Wurzschmitt, he was able to detect only one complex because of the nature of the experimental procedure employed. From his analytical data this product appears to correspond to the second complex of this study which forms only at higher iodine concentrations.

TABLE I.—DATA SHOWING PRECIPITATION OF IODINE AND IODIDE BY POLYETHYLENE GLYCOL IN THE PRIMARY PLATEAU REGION

T = 30° C. Initial PEG Concentration = 0.227 Equiv. Per Liter

Amount of Iodine in Moles Per Liter × 10^3			Amount of Iodide in Equiv. Per Liter × 10^3			
Added	Found in Solution	Precipitated	Added	Found in Solution	Precipitated	Ppt. Iodide / Ppt. Iodine
7.76	0.01	7.75	117.5	110.3	7.2	0.93
10.58	0.01	10.57	117.5	107.9	9.6	0.91
17.22	0.01	17.21	117.5	101.3	16.2	0.94
19.10	0.01	19.09	117.5	99.7	17.8	0.93
21.41	0.01	21.40	117.5	97.7	19.8	0.93
24.38	0.01	24.37	117.5	96.1	21.4	0.88

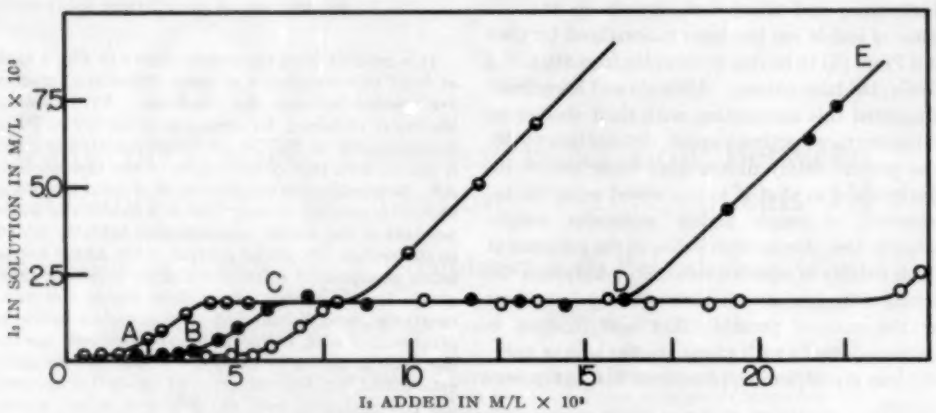

Fig. 1.—A plot showing the precipitation of iodine in the presence of PEG 6000 and 0.140 *N* KI, at 30°. Initial concentration of PEG:—●—13.7 × 10^{-3} equivalents per liter, ●—29.7 × 10^{-3} equivalents per liter, ○—45.1 × 10^{-3} equivalents per liter.

slope of the DE portion of the curve is one, indicating that any further addition of free iodine to the system in this region results in a corresponding increase of iodine in solution, the amount of precipitated iodine remaining constant. The stoichiometry of the complex formed may be calculated from any point in this DE region and is exemplified by the following:

Initial PEG concentration	29.7 × 10^{-3} equivalents per liter
Iodine added to the system	19.18 × 10^{-3} moles per liter
Iodine analyzed in solution	4.22 × 10^{-3} moles per liter
Iodine precipitated	14.96 × 10^{-3} moles per liter

$$\frac{\text{Iodine precipitated in moles per liter}}{\text{Initial PEG concentration in equivalents per liter}} = \frac{14.96}{29.7} = \frac{1}{1.98}$$

This stoichiometric ratio of one iodine bound for every two repeating CH_2OCH_2 units of the polymer is nearly quantitative as is indicated in Table II. This exactness suggests a possible analytical method for polyethylene glycol determination based on this reaction.

TABLE II.—DATA SHOWING THE PRECIPITATION RATIO OF IODINE AND POLYETHYLENE GLYCOL IN THE PRESENCE OF EXCESS FREE IODINE

Initial Concn. of PEG in Equiv. per liter × 10^3	Molar Concn. of Iodine added × 10^3	Molar Concn. of Iodine Found in Soln. × 10^3	Molar Concn. of Iodine Precipitated × 10^3	Iodine Precipitated / Equiv. of PEG
13.7	9.81	2.99	6.82	0.497
13.7	11.89	5.03	6.86	0.500
13.7	13.56	6.71	6.85	0.500
29.7	19.18	4.22	14.96	0.505
29.7	21.55	6.25	15.30	0.515
29.7	22.39	7.14	15.25	0.515
45.1	25.20	2.93	22.30	0.495
45.1	28.26	5.09	23.20	0.515
45.1	30.10	7.50	22.60	0.500

Theoretical Rationalization of the Equilibrium Behavior.—The experimental data presented thus far suggested that initially in aqueous solution, potassium and triodide ions interact with PEG to form an insoluble primary complex with an approximate formula of $HO[KI_3 \cdot (-OCH_2CH_2)_{10}]_nOH$. After precipitation of the polymer in the form of this primary complex, a secondary interaction occurs between the precipitated primary complex and molecular iodine resulting in conversion of the primary

complex to the secondary complex having an approximate formula of $HO[KI \cdot ((-OCH_2CH_2)_9I_2)_8]_n$-OH.

The flatness of the initial plateau indicates that the formation of the primary complex, which appears to involve triiodide ion, potassium ion, and PEG, is curiously insensitive to the concentration of the polymeric substance in solution. This may be rationalized by considering the many binding sites available on a single polymer chain. A qualitative mass law expression may be derived from the assumed interaction,

$$n\mathrm{K}^+ + n\mathrm{I}_3^- + (-\mathrm{CH_2OCH_2}-)_i \rightleftarrows \text{Complex (solid)} \quad (1)$$

$$\mathrm{K} = (\mathrm{I}_3^-)^n(\mathrm{K}^+)^n(-\mathrm{CH_2OCH_2}-)_i$$

Since it appears that the stoichiometry of this primary interaction is of the order of one KI_3 for approximately every 10 monomeric units, the value of n would be approximately 14. It can be seen that since

$$\mathrm{K} = (\mathrm{I}_3^-)^{14}(\mathrm{K}^+)^{14}(-\mathrm{CH_2OCH_2}-)_i$$

variations in polymer concentration would exert only slight effects on the equilibrium concentration of iodine. This behavior and explanation is similar to that observed and used by Higuchi and Lach for the PEG-phenobarbital interaction (1). If this assumption is correct, the equilibrium may be described by an apparent equilibrium quotient,

$$\mathrm{K}' = (\mathrm{I}_3^-)(\mathrm{K}^+) \quad (2)$$

The slight positive slope of the primary plateau, Fig. 2, apparently indicates this slight dependency of the interaction on the polymer concentration. Precipitation of potassium and triiodide ions in this region, however, would also contribute to a positive slope.

An equation and equilibrium quotient can be written, in a similar fashion, representing the assumed transformation of the precipitated primary complex to the secondary complex in presence of excess iodine,

$$n\mathrm{I}_2 + \text{complex I (Solid)} \rightleftarrows \text{complex II (Solid)} \quad (3)$$

$$\mathrm{K}'' = \frac{1}{(\mathrm{I}_2)^n}$$

or

$$\mathrm{K}''' = (\mathrm{I}_2)$$

where $\mathrm{K}''' = \frac{1}{\sqrt[n]{\mathrm{K}''}}$ (4)

Experimental Testing of the Proposed Relationships.—The relationships proposed in the preceding section, explaining the observed phase changes, can be subjected to a limited amount of experimental verification. Thus, for example, if Eq. 1 is a valid representation of the first equilibrium, the concentrations of potassium ion, iodide ion, triiodide ion, and free iodine would be closely interdependent. In a like manner the validity of Eq. 3, describing the second interaction, would be manifested in an interdependency between free iodine, triiodide ion, and iodide ion concentrations. The results of experiments designed to test these aforestated assumptions are presented below and do indicate, as will be seen, that these assumptions are indeed justified.

In the following studies, the total amount of iodine remaining in solution at equilibrium has been used as a reference to evaluate the dependency of the interactions on the various postulated reactants. In order to interpret experimentally obtained data it is first necessary to correlate the concentrations of all iodine containing species existing in solution to the total amount of iodine found in solution by titration. Assuming that at the iodide concentrations employed, iodine exists in solution only as I_2 and I_3^-, we can derive a number of pertinent relationships. Since

$$(\mathrm{I}_2)_t = (\mathrm{I}_2) + (\mathrm{I}_3^-)$$

and

$$\mathrm{I}_3^- \rightleftarrows \mathrm{I}_2 + \mathrm{I}^-$$

$$\mathrm{K} = \frac{(\mathrm{I}_2)(\mathrm{I}^-)}{(\mathrm{I}_3^-)}$$

where

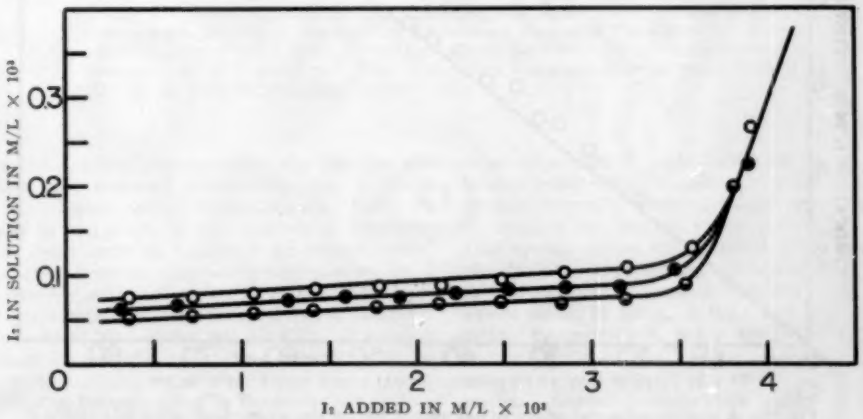

Fig. 2.—A plot showing a magnification of the primary region of Fig. 1 at 30°. Initial concentration of PEG—30.1×10^{-3} equivalents per liter, in the presence of:—○—0.086 *N* KI, ●—0.118*N* KI, ◒—0.155*N* KI.

$(I_2)_t$ = total molar concentration of all iodine containing species,
(I_3^-) = equivalent concentration of triiodide ion and
(I_2) = molar concentration of free iodine,
K = instability constant of the triiodide complex, taken to be in this study 1.4×10^{-3},

the following relationships may be derived,

$$(I_2)_t = (I_3^-) + \frac{K(I_3^-)}{(I^-)}$$

$$= (I_3^-)\left\{1 + \frac{K}{(I^-)}\right\}$$

or

$$(I_3^-) = \frac{(I_2)_t(I^-)}{K + (I^-)} \quad (5)$$

In an analogous manner, it may be shown that

$$(I_2) = \frac{(I_2)_t \cdot K}{K + (I^-)} \quad (6)$$

These preceding relationships may now be substituted in the previously postulated equilibria expressions. Thus, in the case of primary complex formation, the previously postulated equilibrium quotient

$$K' = (I_3^-)(K^+)$$

may now be expressed in terms of known and experimentally determinable quantities, i. e.,

$$K' = \frac{(I_2)_t \cdot (I^-)}{K + (I^-)} \cdot (K^+) \quad (7)$$

The equilibrium quotient for the second interaction, which was previously derived,

$$K''' = (I_2)$$

may now be expressed in a similar manner as

$$K''' = \frac{(I_2)_t \cdot K}{K + (I^-)} \quad (8)$$

Effect of Potassium Ion Concentration on Primary Complex Formation.—A direct relationship between $1/(I_2)_t$ and (K^+) would be expected on examination of the rearranged form of the Eq. 7

$$\frac{1}{(I_2)_t} = \frac{(K^+)(I^-)}{K'\{K + (I^-)\}}$$

This theoretically expected relationship was tested experimentally by (*a*) adjusting a system to the region of the first plateau by an appropriate initial addition of iodine, (*b*) maintaining a constant iodide concentration, and (*c*) noting the effect of varying potassium ion concentrations on the equilibrium concentration of titratable iodine.

Experimental results are shown in Fig. 3. It is seen that $1/(I_2)_t$ is directly proportional to (K^+). Conforming to theoretical expectations, the interaction appears to be first order with respect to potassium ion over relatively wide variations in potassium ion concentration. The points plotted have not been corrected for precipitation of potassium ion. This correction, however, would be small since in most cases the differences between corrected and uncorrected values are negligible compared to the experimental error. The average value of K', uncorrected for precipitation of iodide

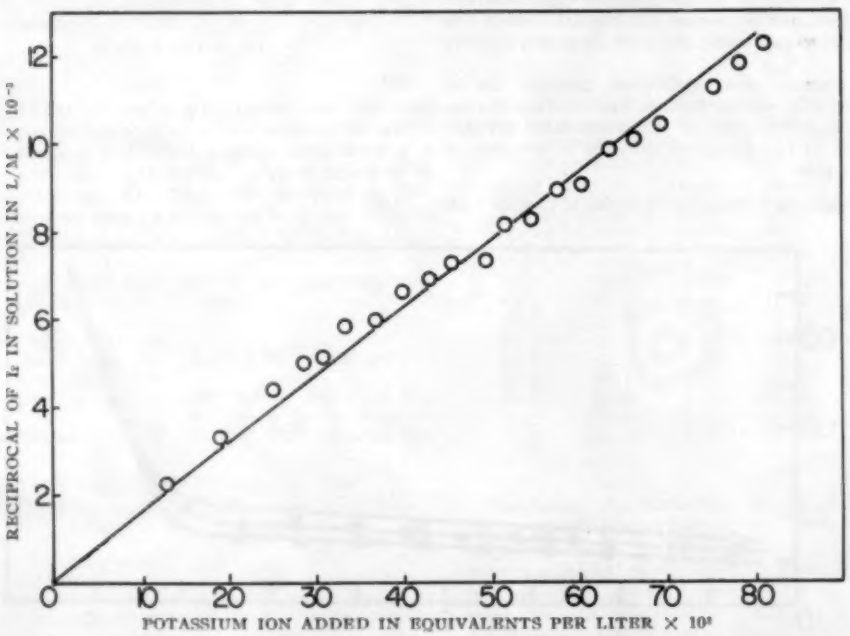

Fig. 3.—A plot showing the effect of potassium ion on the amount of iodine remaining in solution in the region of the primary plateau of Fig. 1 at 30°. Initial concentration of PEG—30.0×10^{-3} equivalents per liter. Initial concentration of iodine—1×10^{-3} moles per liter. Iodide concentration maintained at 82.5×10^{-3} equivalents per liter with NaI.

and potassium ions, calculated from Eq. 7 was found to be 6.14×10^{-6}. It was assumed that the presence of sodium ion in the system had no effect on the equilibrium in question. This assumption was justified by the absence of insoluble complex formation in a corresponding system containing only sodium iodide.

Effect of Iodide Ion Concentration on Primary Complex Formation.—The dependency of the interaction on iodide ion concentration was experimentally determined in a manner parallel to that described above, i. e., (*a*) a system was adjusted to the region of the first plateau by appropriate initial addition of iodine, (*b*) a constant potassium ion concentration was maintained, and (*c*) the effect of varying iodide ion concentration on the equilibrium concentration of iodine in solution was noted. Experimental results are summarized in Fig. 4.

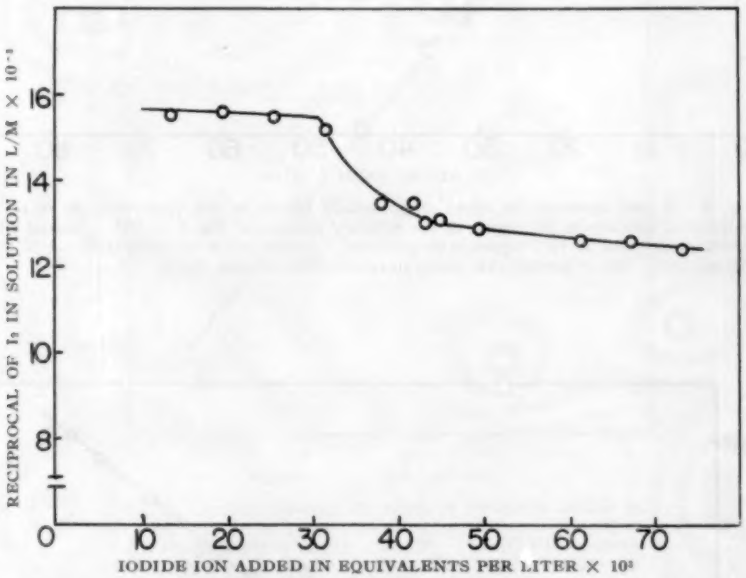

Fig. 4.—A plot showing the effect of iodide ion on the concentration of iodine remaining in solution in the region of the primary plateau of Fig. 1 at 30°. Initial concentration of PEG—30.0×10^{-3} equivalents per liter. Initial concentration of iodine—1×10^{-3} moles per liter. Potassium ion concentration maintained at 82.5×10^{-3} equivalents per liter with KCl.

It can be seen from examination of this plot that the theoretically expected relationship, Eq. 7, holds only at higher iodide concentrations. Here, as would be expected, $1/(I_2)_f$ exhibits a relatively slight dependency on the iodide ion concentration. Deviations from the relationship expressed by Eq. 7 do, however, appear to be significant in systems having an iodide ion concentration below approximately 50×10^{-3} equivalents per liter. A possible explanation for this anomalous behavior will be offered in a subsequent section.

Effect of Potassium Iodide Concentration on Primary Complex Formation.—The combined effect of both potassium and iodide ion variations on the primary interaction was determined experimentally in a manner parallel to that discussed above. Thus, the potassium iodide concentration was varied at a constant salt concentration and the effect of potassium iodide concentration on the interaction was determined by noting its effect on the total amount of iodine remaining in solution after attainment of equilibrium. It is seen from Fig. 5 that experimental results appear to conform to theoretical expectations only at higher potassium iodide concentrations. Similar to the effect observed at lower iodide concentrations, deviations are significantly apparent at lower potassium iodide concentrations. Figure 6 emphasizes these deviations. Here $1/(I_2)_f$ is plotted as a function of $(K^+)(I^-)/(I^-) + K$. This plot, in accordance with Eq. 7, should yield a straight line, passing through the origin, the slope of which should correspond to the reciprocal of K'. A straight line drawn through those experimental points obtained at high potassium iodide concentrations appears to extrapolate through the origin. The value of K' calculated from the slope of this line was found to be 6.22×10^{-6}. This agrees, within experimental error, with previously determined values. It is seen from Fig. 6 that pronounced deviations from the expected behavior occur in those systems having potassium iodide concentrations lower than approximately 55.0×10^{-3} molar. It thus appears that these deviations depend in some way on the iodide concentration. A possible explanation will be offered in a subsequent section.

Effect of Potassium Iodide Concentration on Secondary Complex Formation.—The theoretically

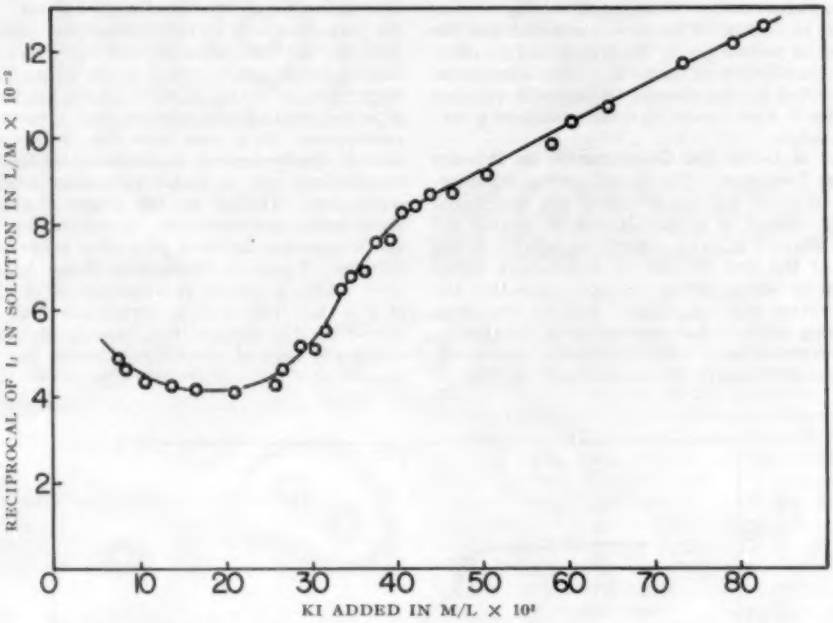

Fig. 5.—A plot showing the effect of potassium iodide on the concentration of iodine remaining in solution in the region of the primary plateau of Fig. 1 at 30°. Initial PEG concentration—30.0 × 10^{-3} equivalents per liter. Initial iodine concentration—1 × 10^{-3} moles per liter. Salt concentration maintained at 0.0825 *N* with NaCl.

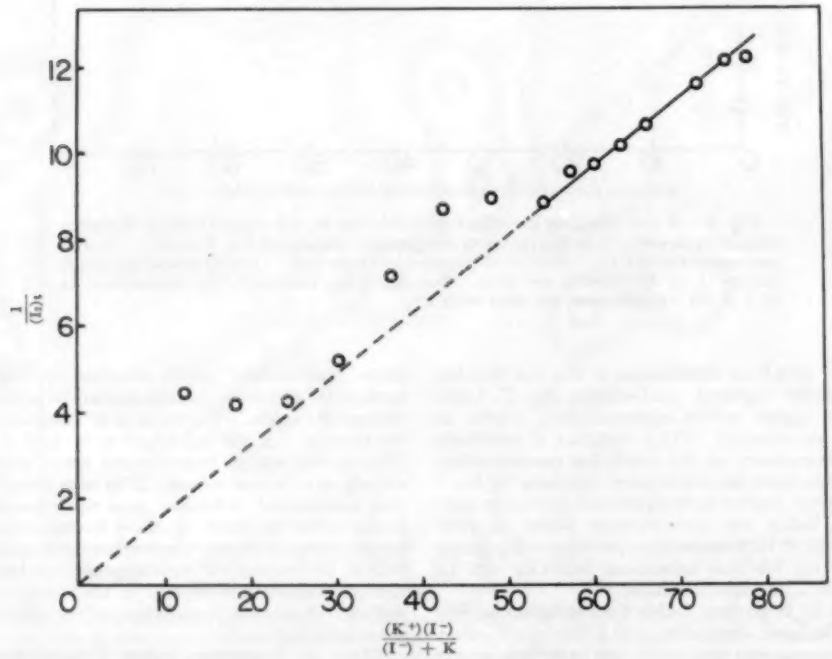

Fig. 6.—A plot showing the data from Fig. 5 plotted in accordance with Eq. 7.

expected linear relationship between iodide ion concentration and total titratable iodine in the region of the second plateau, i.e.,

$$(I_2)_t = K''' + \frac{K'''}{K}(I^-),$$

was tested experimentally by noting the effect of varying iodide concentrations on the total amount of iodine remaining in solution in a system in this region. Experimental results are summarized in Fig. 7. Here, the molar concentration of titrated iodine is plotted as a function of the potassium iodide concentration. It is seen that the theoretically expected relationship holds over the concentration range studied. The average value of K''', uncorrected for precipitation of iodide, calculated from individual points was found to be 1.74×10^{-4}. This value agrees well with the value of 1.72×10^{-4} calculated from the slope of the straight line of Fig. 7. This experimental evidence would indicate then, that the assumed potassium ion independent interaction, Eq. 3, is a logical one.

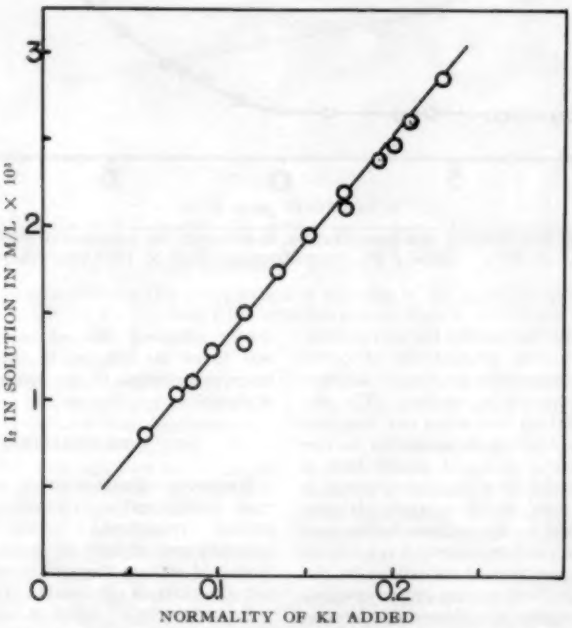

Fig. 7.—A plot showing the effect of potassium iodide on the concentration of iodine remaining in solution in the region of the second plateau of Fig. 1 at 30°. Initial PEG concentration—30.0×10^{-3} equivalents per liter. Initial iodine concentration—8.67×10^{-3} moles per liter.

It is now apparent that two consecutive competitive interactions result in the removal of iodine from solution. It has been shown that a primary interaction results in the precipitation of iodine, apparently as $HO[KI_3 \cdot (—OCH_2CH_2)_{10}]_nOH$, from solution. A second interaction, it has been shown, then occurs between free iodine and the precipitated primary complex, if the free iodine concentration exceeds a critical value, removing molecular iodine from solution. Since the concentration of iodide ion determines the concentration of free iodine according to the mass law expression,

$$K = \frac{(I^-)(I_2)}{(I_3^-)},$$

relatively high iodide ion concentrations apparently fix the free iodine concentration at values below that required for the secondary interaction to occur. In these cases behavior theoretically expected from Eq. 7 is apparent. Free iodine concentrations do, however, appear to exceed the critical value at lower iodide ion concentrations, resulting in precipitation of iodine due to the secondary interaction as well as the primary interaction. Coprecipitation of triiodide ion and free iodine thus appears to manifest itself in the aforementioned apparent anomalies. Although experimentally determined points do not agree too closely with those predicted by a combination of Eq. 7 and 8, the expression obtained does describe qualitatively the general shape of the experimentally determined curves. Formation of iodine-iodide complexes of higher iodine order might possibly complicate the system at lower iodide concentrations.

OTHER SYSTEMS STUDIED

Iodine-Sodium Iodide PEG.—Figure 8 illustrates a phase study analogous to that described in Fig. 1 carried out in the presence of sodium iodide. It can

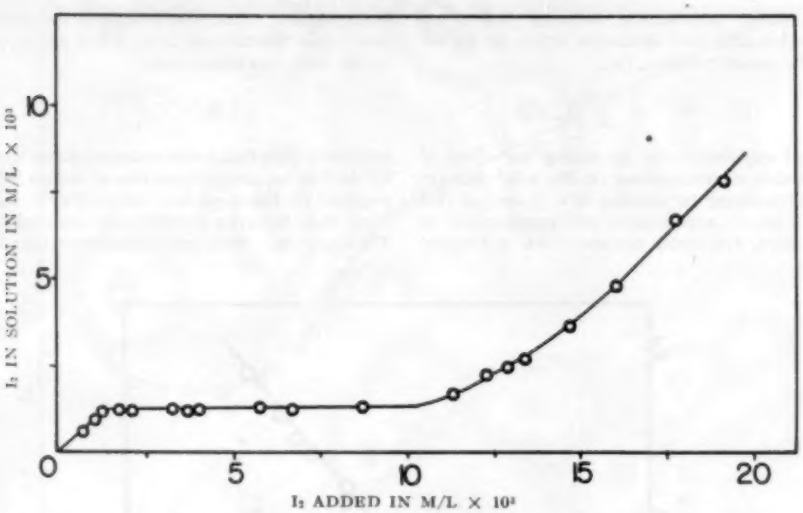

Fig. 8.—A plot showing the precipitation of iodine in the presence of PEG 6000 and 0.100 *N* NaI at 30°. Initial PEG concentration—30.0 × 10^{-3} equivalents per liter.

be seen from this figure that unlike the corresponding potassium iodide system, precipitation of iodine occurs only when a comparatively higher concentration of iodine in solution is reached. The absence of a primary plateau and clean cut stoichiometric ratio indicates marked dissimilarity to the potassium iodide system. It would appear from a comparison of Fig. 1 and 8 that no primary complex is formed in the sodium iodide system. It was apparent, however, that in the sodium iodide system as in the potassium iodide system, a red-brown colored insoluble complex formed initially. In the former case, at the specified sodium iodide concentration, the primary complex was observed to be converted immediately after formation to the black secondary insoluble complex. It was qualitatively observed from simple test tube studies that the corresponding lithium iodide system exhibited similar behavior. It is confusing to note that in the sodium iodide system the saturation stoichiometric ratio appears to be one iodine bound to every three repeating monomeric units. This differs considerably from the potassium iodide system in which the saturation stoichiometric ratio was found to be one iodine bound to every two repeating units. No attempt has been made to rationalize the differences encountered with different alkali cations.

Bromine-Potassium Bromide PEG.—It is interesting to note that bromine in the presence of bromide ions also interacts reversibly with PEG in aqueous media to form insoluble complexes. It can be seen from Fig. 9 that the bromine interaction appears to simulate the iodine interaction in a qualitative sense. Although marked differences from the iodine system are apparent no rigorous experimental investigation of the bromine interaction has been attempted. The yellow-white bromine complex was observed to be readily converted to the red-brown iodine complex by the addition of potassium iodide to the aqueous system. The bromine complex was found to be extremely unstable in air, slowly releasing gaseous bromine. This complex was found to behave, in aqueous medium, as a bromine reservoir in, for example, the bromination of phenol.

EXPERIMENTAL

Reagents.—Reagent grade sodium iodide, potassium iodide, sodium chloride, potassium chloride, phenol, resublimed iodine, reagent bromine, polyethylene glycol[1] of average mol. wt. 6,000. Standard sodium thiosulfate solution 0.1 *N*, standard silver nitrate solution 0.1 *N*.

Procedure.—(*a*) *Effect of Iodine Concentration.*—Varying weights of resublimed iodine were weighed into narrow-necked, glass-stoppered, 125-ml. soft-glass bottles, containing 50 ml. of a stock solution of PEG and an alkali iodide.[2] The bottles were mechanically agitated at 30° for at least five hours. The insoluble complexes formed were allowed to settle and 20-ml. aliquots of the clear supernatant liquid were withdrawn for iodine analysis. Iodine was determined by semimicro titration with 0.01 *N* sodium thiosulfate, potentiometrically using a platinum indicator electrode for concentrations lower than 1 × 10^{-3} *M*, and with starch indicator for concentrations exceeding 1 × 10^{-3} *M*.

(*b*) *Effect of Potassium Concentration on Primary Complex Formation.*—Into a series of glass-stoppered, narrow-necked, 125-ml. bottles were pipetted or buretted, 45 ml. of an aqueous stock polymer solution, 5 ml. of a stock iodine solution in 0.150 *N* potassium iodide, and varying volumes of 0.150 *N* potassium iodide. A total volume of 100 ml. was made, in each case with 0.150 *N* sodium

[1] Distributed by the Carbon and Carbide Chemical Company under the trade name of Carbowax® 6,000.

[2] The appearance of brown coloration in stock polyethylene glycol–alkali iodide aqueous solutions after approximately twelve hours indicated oxidative decomposition of the polyether. For this reason all PEG stock solutions were prepared fresh immediately before use.

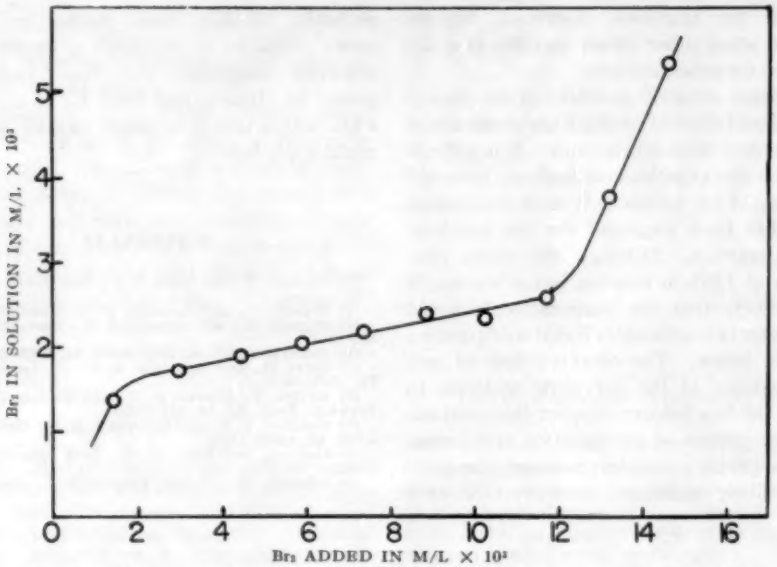

Fig. 9.—A plot showing the precipitation of bromine in the presence of PEG 6000 and 0.150 *N* KBr at 0°. Initial PEG concentration—30.0 × 10^{-3} equivalents per liter.

iodide. In this manner an approximately constant iodide ion concentration was maintained at 0.0825 *N* while variations in potassium ion concentration could be made from approximately 0.008 *N* to 0.0825 *N*. The bottles were agitated at 30°, and when equilibrated, aliquots of the clear supernatant liquid were withdrawn for iodine analysis as previously outlined.

(*c*) *Effect of Iodide Ion Concentration on Primary Complex Formation.*—In a manner analogous to (*b*), approximately constant potassium ion concentration was maintained at 0.0825 *N*, while iodide ion concentration was varied from approximately 0.008 *N* to 0.825 *N*, using 0.150 *N* solutions of potassium iodide and potassium chloride. In a like manner the bottles were agitated, equilibrated and aliquots analyzed for iodine content.

(*d*) *Effect of Potassium Iodide Concentration on.*—*1. Primary Complex Formation.*—In a manner similar to (*b*) and (*c*) variations in potassium iodide concentrations were made at an approximately constant total salt concentration of 0.0825, using 0.150 *N* solutions of potassium iodide and sodium chloride. *2. Secondary complex formation.*—Possible complication from cations other than potassium prevented maintenance of constant salt concentration. In this case, varying volumes of potassium iodide stock solution were added to 45 ml. of stock polymer solution and 5 ml. of a stock iodine–potassium iodide solution. A total volume of 100 ml. was made up with distilled water. It was observed that below total salt concentrations of approximately 0.05 *N*, the precipitates were no longer well defined but remained colloidally suspended, preventing withdrawal of clear aliquots. Investigation in this case was thus confined to potassium iodide concentrations exceeding approximately 0.050 *N*.

(*e*) *Iodide Analysis.*—Iodine was first removed from an iodine-iodide solution adsorptively with Norite®. Iodide was then determined titrimetrically with silver nitrate using eosin as the adsorption indicator.

(*f*) *Qualitative Test for the Presence of Polyethylene Glycol in Solution.*—A saturated solution of phenol in water was added dropwise to a portion of the supernatant liquid after thiosulfate treatment. Permanent or transient cloudiness was indicative of the presence of Carbowax® in solution.

(*g*) *Bromine-PEG Interaction.*—Into each of a series of 125-ml. narrow-necked, glass-stoppered, soft-glass bottles was pipetted 25 ml. of a stock solution of Carbowax® 6000 in distilled water. Various appropriate volumes of a stock solution of bromine in 0.3 *N* KBr were buretted into the bottles. A final volume of 50 ml. was made in each case with 0.3 *N* KBr. All solutions were well cooled in an ice bath prior to use. The bottles were stoppered and mechanically agitated for twelve hours at 0°. The solid complexes formed were then allowed to settle and aliquots of the clear supernatant liquid withdrawn for analysis. The bromine content was determined by adding KI to the aliquot and analyzing the released iodine with 0.01 *N* $Na_2S_2O_3$ using starch indicator.

CONCLUSIONS

Although the experimental findings, presented above, are insufficient bases for the total elucidation of the nature of these complexes, they do, however, give us an interesting partial picture of rather unusual systems. Despite the fact that ethers are generally considered to be relatively unreactive molecular species, we find in this system a polyether being precipitated out of solution by potassium and triiodide ions. The

nature of the reactions, moreover, appears to change when other alkali metallic ions are substituted for potassium ions.

Information obtained as results of the present studies is insufficient to establish the structures of the products of these interactions. It is difficult to interpret the experimental findings, however, on the basis of an occlusion type of mechanism such as has been suggested for the amylose-iodine interaction. Although the exact configuration of PEG in solution is not known, it seems unlikely that the macromolecule would exist in water in a sufficiently coiled configuration to occlude iodine. The observed lack of any strong tendency of the polymeric molecule to solubilize the free halogen support this position. During the process of precipitation and formation of the primary complex, however, the polymer very likely undergoes *quasi* crystallization probably through some coiling process. It seems probable, on the basis of experimentally observed similarities, that those factors suggested by Higuchi and Lach (1) to explain the PEG interaction with small organic molecules might apply here.

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Preparation and Properties of Citric and Tartaric Acid Salts of Piperazine*

By J. J. HEFFERREN, G. SCHROTENBOER, and W. WOLMAN

The following salts of piperazine with tartaric and citric acids were prepared: piperazine monotartrate, piperazine ditartrate, piperazine monocitrate, piperazine dicitrate, and tripiperazine dicitrate. There are indications that dipiperazine monotartrate and tripiperazine monocitrate exist in solution, but attempts to isolate these salts were not successful. The tartaric and citric acid salts of piperazine are odorless white crystalline solids with relatively high water solubility. The tartrate salts are nonhygroscopic whereas the citrate salts occur as a variety of hydrates, some of which are quite stable. All the salts are relatively stable at elevated temperatures and are suitable for incorporation in common pharmaceutical forms; however, there are characteristic differences which should be considered in their use.

AS EARLY AS 1906, piperazine and a methyl derivative were asserted to be efficacious remedies in the treatment of gout and rheumatism (1). With the successful introduction of diethylcarbamazine hydrochloride (1-diethylcarbamyl-4-methylpiperazine hydrochloride) in the treatment of filariasis (2), piperazine and its various derivatives were screened for anthelmintic activity. In 1951, piperazine hexahydrate was used successfully in the treatment of oxyuriasis in children (3). The citric and tartaric acid salts of piperazine are now accepted in the treatment of infections caused by pinworms (*Enterobius vermicular; Oxyuris vermicularis*) and roundworms (*Ascaris lumbricoides*) (4).

* Received May 5, 1955, from the Chemical Laboratory of the American Medical Association, Chicago, Ill.

Commercially available piperazine is in the form of the hexahydrate which is a hygroscopic, deliquescent, strongly basic, crystalline solid with a low melting point and amine-like odor. These characteristics were strong deterrents for the general use of the hexahydrate in medicine. Attempts to prepare more suitable forms of piperazine for incorporation into common pharmaceutical dosage forms have resulted in the preparation of various carboxylic acid salts of piperazine. Included in the list of these salts are: piperazine diphenylacetate (5), piperazine monoadipate (6), piperazine citrate (7), and piperazine tartrate (8). At the time this study was initiated the only isolated salt of piperazine that could be found described in the literature was piperazine diphenylacetate. During the course of the

study piperazine monoadipate was described (6) and tripiperazine dicitrate was prepared (7). Piperazine monocitrate was mentioned in a pharmacological and toxicity report (7).

One of the first commercially available preparations of piperazine was a syrup of piperazine citrate. During the course of a chemical study of this drug, it became evident that several salts of piperazine and citric acid were theoretically possible. If this proved to be true, a knowledge of the properties of these salts would be of value in the consideration of problems of formulation and therapeutics. A study of the conditions that govern the formation of the various salts was undertaken.

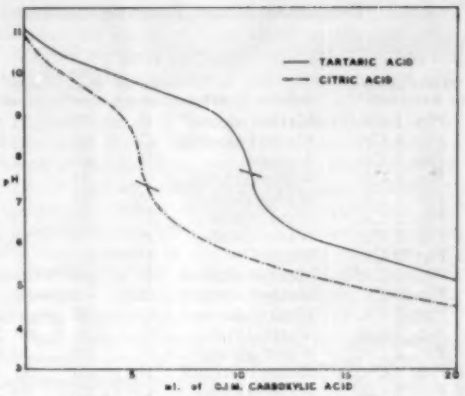
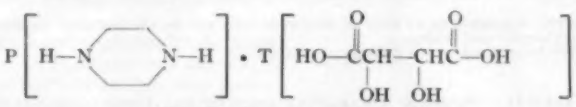


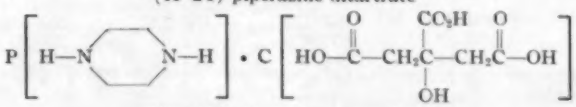
Fig. 1.—Piperazine *vs.* carboxylic acid.

DISCUSSION

By using different conditions two salts of piperazine and tartaric acid and three salts of piperazine and citric acid were prepared and isolated. The salts were piperazine monotartrate, piperazine ditartrate, piperazine monocitrate, piperazine dicitrate, and tripiperazine dicitrate.



(1P-1T) piperazine monotartrate
(1P-2T) piperazine ditartrate



(1P-1C) piperazine monocitrate
(1P-2C) piperazine dicitrate
(3P-2C) tripiperazine dicitrate

Potentiometric titration of piperazine with tartaric or citric acid revealed single breaks in the curves corresponding to the theoretical combinations, dipiperazine monotartrate and tripiperazine monocitrate (Fig. 1). Attempts to isolate these salts were not successful.

Several factors were considered that might conceivably influence the preparation of the different carboxylic acid salts. The effect of varying the molar ratios and the order of addition of the reactants as well as temperature and reaction media were investigated.

The salt isolated from the reaction of piperazine and tartaric acid was dependent entirely upon the molar ratios of the reactants. In all cases where piperazine was present in at least equal molar quantities with tartaric acid, piperazine monotartrate was isolated. When the tartaric acid was present in at least a twofold molar excess, piperazine ditartrate was isolated. In the aqueous reaction of 2 moles of piperazine hexahydrate with 3 moles of tartaric acid, two salts were isolated, piperazine monotartrate and piperazine ditartrate.

The salt isolated from the reaction of piperazine and citric acid was dependent upon the molar ratios of the reactants, solvent system, as well as the temperature of the reaction mixture (Table I). Piperazine dicitrate could not be isolated from nonaqueous reaction media at either room temperature or 65°, whereas tripiperazine dicitrate could not be obtained in nonaqueous media at 65°.

Physical Properties.—All five salts of piperazine

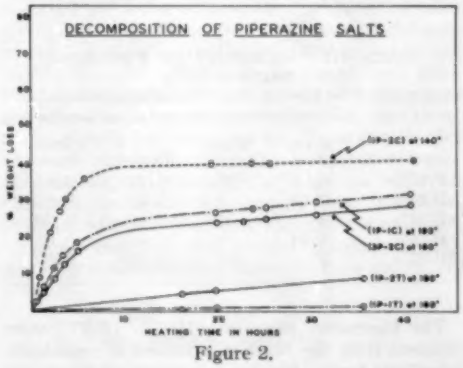


Figure 2.

with citric and tartaric acids are white, odorless, crystalline solids with an acid taste. The tartrates and anhydrous citrates melt with decomposition and evolution of gas. The *p*H of the salt solutions decreases as the proportion of the acid part of the molecule increases (Table II).

TABLE I.—EFFECTS OF MOLAR RATIO OF THE REACTANTS, SOLVENT SYSTEM, TEMPERATURE, AND ORDER OF MIXING

Molar Ratio of Reactants[a]	Solvent System[b]	Order of Mixing[c]	Temp. of Reaction°	%, Piperazine[d] Calcd.	%, Piperazine[d] Found	m. p.° (uncorr.[e])	Salt Isolated	Weight Loss at 105° for 4 Hrs., %	Yield, %
1 Pip. 1 Cit.	Methyl alcohol	P	25	31.0	30.4	205–206 D	(1P-1C)	1.6	..
1 Pip. 1 Cit	Methyl alcohol	C	65	31.0	30.7	205–206 D	(1P-1C)	1.3	94
1 Pip. 1 Cit.	Acetone	P	25	31.0	29.9	206–208 D	(1P-1C)	..	83
1 Pip. 1 Cit	Water-alcohol	C	25	31.0	30.9	202–203 D	(1P-1C)	1.5	96
1 Pip. 1 Cit.	Water-alcohol	P	25	31.0	30.7	203–204 D	(1P-1C)	1.1	94
1 Pip. 2 Cit.	Water-alcohol	C	25	18.3	17.3	163–170 D	(1P-2C)	13.5	84
1 Pip. 2 Cit.	Water-alcohol	P	25	18.3	17.5	161–168 D	(1P-2C)	13.3	83
1 Pip. 2 Cit.	Water	P	25	18.3	17.8	168–170 D	(1P-2C)	13.1	..
1 Pip. 3 Cit.	Methyl alcohol	P	25	31.0	29.7	205–207 D	(1P-1C)	1.2	88
1 Pip. 3 Cit.	Methyl alcohol	C	65	31.0	30.7	205–206 D	(1P-1C)	1.3	83
3 Pip. 2 Cit.	Methyl alcohol	C	25	40.2	39.6	184–187 D	(3P-2C)	13.0	88
3 Pip. 2 Cit.	Water-alcohol	C	25	40.2	38.6	183–186 D	(3P-2C)	12.2	96
3 Pip. 2 Cit.	Water-alcohol	P	25	40.2	38.3	183–186 D	(3P-2C)	10.8	94
3 Pip. 2 Cit.	Methyl alcohol	P	65	31.0	30.2	205–206 D	(1P-1C)	1.3	..
2 Pip. 1 Cit.	Water-alcohol	C	25	40.2	39.9	185–187 D	(3P-2C)	11.8	98
2 Pip. 1 Cit.	Water-alcohol	P	25	40.2	39.7	185–187 D	(3P-2C)	11.6	90
2 Pip. 1 Cit.	Methyl alcohol	P	25	40.2	39.2	183–186 D	(3P-2C)	12.6	94
2 Pip. 1 Cit.	Acetone	P	25	40.2	39.3	180–187 D	(3P-2C)	11.7	87
2 Pip. 1 Cit.	Methyl alcohol	P	65	31.0	30.5	205–206 D	(1P-1C)	0.9	60
2 Pip. 1 Cit.	Methyl alcohol	C	65	31.0	30.3	205–206 D	(1P-1C)	0.5	..
3 Pip. 1 Cit.	Water-alcohol	C	25	40.2	39.9	183–185 D	(3P-2C)	11.6	97

[a] The piperazine was added as piperazine hexahydrate, while the citric acid was added as the monohydrate (analytical reagent).

[b] In nonaqueous media, respective solutions of piperazine and citric acid were mixed, and the resultant precipitate removed by suction filtration. In hydroalcoholic media, aqueous solutions of the reactants were mixed, and the product was precipitated from the solution with the addition of either methyl or ethyl alcohol.

[c] Order of Mixing: P, Piperazine was added to the citric acid solution. C, Citric acid was added to the piperazine solution.

[d] The percentage piperazine represents an average of determinations run on the material obtained after simple washing with either methyl or ethyl alcohol and drying.

[e] All melting points were determined on the anhydrous material.

TABLE II.—PHYSICAL CHARACTERISTICS OF THE PIPERAZINE SALTS

	(1P – 1C)	(1P – 2C)	(3P – 2C)	(1P – 1T)	(1P – 2T)
M. p.	203–208°	167–172°	183–187°	258–263°	237–243°
Mol. wt.	278.27	470.40	642.68	236.23	386.32
Equiv. wt.	139.14	94.08	214.23	236.23	128.77
*p*H of 1% solution	4.7	3.6	5.5	4.8	3.5

The piperazine salts of citric acid exhibit typical salt-like solubility. They are very soluble in water and are insoluble in organic solvents (Table III).

TABLE III.—SOLUBILITY OF PIPERAZINE SALTS AT 25°

	Gm. of Solute per 100 cc. of Solution			
	Gl. Acetic Acid	Water (Approx.)	Alcohol	Chloroform
(1P-1T)	0.07	26	0.01	0.01
(1P-2T)	0.03	1	0.01	0.01
(1P-1C)	4	60	0.02	0.02
(1P-2C)	0.04	10	0.06	0.01
(3P-2C)	3	60	0.01	0.02

The piperazine tartrates (1P-1T, 1P-2T) were obtained from the reaction mixtures in essentially anhydrous form. Neither salt exhibited significant change in weight or physical characteristics when placed in an atmosphere of 81% humidity at 25° for as long as ninety-six hours.

Each of the three piperazine citrate salts appeared to have different hydration characteristics. Tripiperazine dicitrate, isolated from the preparative reactions, was usually in the form of the tetra- or pentahydrate. When the hydrate or the anhydrous salt was placed in an atmosphere of 81% humidity at 25°, there was an increase in weight as much as 40%. This additional water was quickly lost when the salt was placed in an atmosphere of lower humidity. Piperazine monocitrate, as isolated from the reaction mixture, was essentially anhydrous. When the salt was placed in an atmosphere of 81% humidity at 25°, the degree of hydration rapidly reached a stable condition, which was approximately equivalent to one molecule of hydration. Piperazine dicitrate was isolated from the preparative reactions in a degree of hydration roughly equivalent to $3\frac{1}{2}$ molecules of water. A loss in weight of about 1% occurred when this product was placed over phosphorus pentoxide in a desiccator that was evacuated for twenty-four hours with a vacuum pump followed by an additional seventy-two hours of standing in the evacuated atmosphere. On the other hand, heating at 105° for four hours caused a loss in weight of about 13%, while the Karl Fischer determination indicated about 13% moisture. When this hydrated piperazine dicitrate was placed in an atmosphere of 81% humidity at 25°, the percentage moisture gradually increased to about 15% where it remained fairly constant for one hundred and twenty hours. Anhydrous piperazine dicitrate was pre-

pared by heating the hydrate in a vacuum oven at 60° for twelve hours. When this salt was placed in an atmosphere of 81% humidity at 25°, there was a gradual increase in weight of about 15%. After this increase there was little change for two hundred hours. The 15% weight increase is approximately equal to four molecules of water of hydration. Thus it appears that piperazine dicitrate forms a relatively stable tetrahydrate. In contrast to the hydration states of piperazine monocitrate and tripiperazine dicitrate, this water of hydration can be removed only at elevated temperatures.

Heating both tartrate salts (1P-1T, 1P-2T) at 105° for as long as one hundred and nine hours resulted in no significant change in the weight, melting point, or physical characteristics of the salts. When piperazine monotartrate salt was heated at 180° for thirty-six hours, there was a gradual loss in weight of about 1.4% (Fig. 2). During this period of heating the solid turned light tan in color within the first few hours and showed very little physical change thereafter. When piperazine ditartrate was heated at 180° for thirty-six hours, there was a gradual weight loss of about 9.7%. During the first hour, the white salt turned tan in color and then the color rapidly deepened to a dark brown. The decomposition of piperazine ditartrate like that of piperazine monotartrate appeared to take place according to an approximate straight-line relationship as shown in Fig. 2.

Piperazine monocitrate and tripiperazine dicitrate exhibited little change in their physical characteristics when heated at 105° for seventy-two hours. Piperazine dicitrate very gradually lost a small percentage of its weight without any apparent change in the physical characteristics when heated at 105° for prolonged periods. A very rapid loss of weight occurred within the first ten hours (Fig. 2) when piperazine monocitrate and tripiperazine dicitrate were heated at 160° and piperazine dicitrate was heated at 140°. After the first period of rapid change, there was a very gradual loss of weight. An attempt was made to determine the correlation, if any, between the loss in weight of the salts and the carbon dioxide evolved, using an apparatus for the evolution and distillation of carbon dioxide (9). After the samples were heated at 200–210° in a vacuum for thirty-six hours, the carbon dioxide evolved was equivalent to the weight loss of the salts when heated at 140 and 160° at atmospheric pressure for twenty-five hours. This percentage of carbon dioxide evolved was roughly equivalent to the loss of two molecules of carbon dioxide by each mole of citric acid in the piperazine mono- and dicitrates. No attempt was made to isolate the decomposition products.

Chemical Properties.—Potentiometric titration of the citrate salts with 0.1 *N* perchloric acid in glacial acetic acid revealed one distinct break in the curve, which was equivalent to the neutralization of both nitrogens of piperazine in the salt. The salts were dissolved in glacial acetic acid by heating on a steam bath. The solution was cooled to room temperature and titrated potentiometrically with either calomel or silver chloride and glass electrode systems or titrated colorimetrically using 1% crystal violet in glacial acetic acid as an indicator.

Piperazine monotartrate was titrated with perchloric acid by first dissolving the salt in 1 ml. of water and then adding the acetic acid. By using an appropriate blank, the salt could be titrated either potentiometrically or colorimetrically. Due to the limited water-solubility of the ditartrate salt, this method was not successful.

Potentiometric titration of tartaric and citric acids with 0.1 *N* sodium hydroxide revealed one break in the curve corresponding to the neutralization of two and three carboxylic acid groups, respectively. When piperazine monotartrate and monocitrate were titrated with 0.1 *N* sodium hydroxide, single breaks in the curves were exhibited corresponding to the neutralization of one and two carboxylic acid groups, respectively (Fig. 3). Similar titration of the other salts (1P-2T, 1P-2C, 3P–2C) revealed breaks in the curves corresponding to neutralization of three, five, and three carboxylic acid groups, respectively.

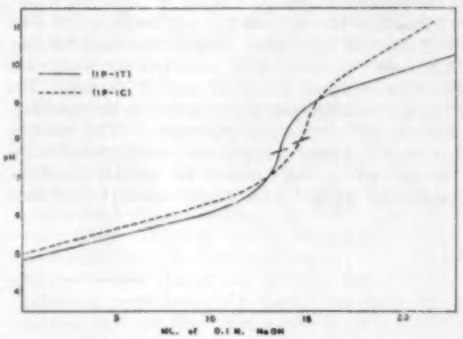


Fig. 3.—Piperazine salts *vs.* NaOH.

Potentiometric titration of piperazine hexahydrate with 0.1 *N* hydrochloric acid revealed two distinct breaks in the titration curve at about *p*H 8 and 4. Thus there is a considerable difference in the basicity of the two nitrogens of piperazine. If it is assumed that only one of the two piperazine nitrogen atoms is sufficiently basic to unite with a carboxylic acid group in a combination that is resistant to cleavage by 0.1 *N* sodium hydroxide, the titration values can readily be explained. Piperazine monocitrate has a total of three carboxylic acid groups with two nitrogen atoms, one of which is strongly basic; therefore, titration with 0.1 *N* sodium hydroxide reveals two titratable carboxylic acid groups. Piperazine monotartrate has two carboxylic acid groups with two nitrogens, one of which is strongly basic; therefore, titration with 0.1 *N* sodium hydroxide reveals one titratable carboxylic acid group. Similarly the observed titration values of the other salts can be explained.

EXPERIMENTAL

Reagents.—The tartaric acid and citric acid monohydrate used were analytical reagent grade. The piperazine hexahydrate used satisfied the requirements of "Tests and Standards for New and Nonofficial Remedies" (10).

Procedure A.—In two suitable flasks, 3.9 Gm. (0.02 mole) of piperizine hexahydrate and 4.2 Gm. (0.02 mole) of citric acid monohydrate were each

dissolved in 3 ml. of water using heat if necessary. The solutions were cooled to room temperature, and the citric acid solution added to the mechanically stirred piperazine solution. The mixture was stirred at room temperature for about thirty minutes. About 50 ml. of methyl alcohol was added, and the mixture was cooled to complete precipitation. The precipitate was removed by suction filtration, thoroughly washed with methyl alcohol, and then air dried. Piperazine monocitrate, melting with decomposition at 203–208° was obtained in about 95% yield. The other piperazine carboxylic acid salts were obtained using the same method with appropriate molar ratios of the reactants. In the preparation of the piperazine carboxylic acid salt composed of two moles of acid and one mole of piperazine, it was preferable to keep the acid concentration in the reaction mixture high, thus the piperazine solution should be added to the solution of the carboxylic acid.

Procedure B.—In two 150-ml. beakers, 3.9 Gm. (0.02 mole) of piperazine hexahydrate and 4.2 Gm. (0.02 mole) of citric acid monohydrate were each dissolved in about 35 ml. of methyl alcohol. The citric acid solution was slowly added to the mechanically stirred solution of piperazine. The mixture was stirred at room temperature for thirty minutes. The precipitate was removed by suction filtration, thoroughly washed with methyl alcohol, and then air dried. Piperazine monocitrate, melting with decomposition at 203–208°, was obtained in about 95% yield. In the preparation of piperazine ditartrate, it was preferable to add the piperazine to the tartaric acid solution. Piperazine dicitrate could not be prepared by this method. The other piperazine carboxylic acid salts were obtained using the same method with appropriate molar ratios of the reactants.

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Bacteria-Excluding Filters for Oils*

By KENNETH E. AVIS and LOUIS GERSHENFELD†

Various unglazed porcelain and diatomaceous earth candles, sintered glass filters, and asbestos pad filters were studied under prolonged contact with corn oil to determine whether or not such filters were effective. Tests of their physical properties and, especially, of their ability to retain bacteria and spores before and after prolonged contact with the corn oil were performed. The results of these tests are presented.

FILTERS have been used for the sterilization of oils for quite some time. Reports have appeared in the literature which have indicated that oils can be sterilized by filtration through filters of adequate porosity (1–3). However, it also has been reported (4) that filters become more permeable to bacteria when they are coated with an oil.

Although published reports, as indicated above, have varied, filters are currently being used for the sterilization of fixed oils intended for use as vehicles for parenteral preparations. This fact was noted also from replies in a survey questionnaire sent to 59 American manufacturers of parenteral products, 52 of whom (88 per cent) responded. Among the questions asked was, "Do you use bacteria-excluding filters for sterilizing oil or oleaginous solutions?" Of the 39 manufacturers who answered this question, 11 indicated that they did use bacteria-excluding filters for sterilizing oils or oleaginous solutions. However, some of them did not rely upon this method but also heat-sterilized the oil or oleaginous solution either before or after filtration. These observations resulted in this study.

Particular attention has been given to the candle-type filters made of either unglazed porcelain or of diatomaceous earth. A sintered glass Buchner filter and an asbestos pad filter were

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also included. Preliminary observations of the flow rates through candles and of the carbon dioxide-oil bubbling pressures with corn oil, cottonseed oil, and sesame oil have shown no significant differences with these different oils. Accordingly, only one oil (corn) was selected to be used throughout the remainder of this study.

EXPERIMENTAL

A series of 18 different filters were set up for vacuum filtration. Corn oil was passed through, alternating vacuum and gravity filtration, for a continuous period of 84 days. At the beginning of the filtration test period, in about the middle, and at the end, the rate was determined at which the oil flowed through the candles in ml. per hour under a vacuum of approximately 5 mm. of Hg. The pressure at which bubbles of carbon dioxide emerged from the candles (a test for pore size) when they were immersed in distilled water and in corn oil was determined prior to the filtration test, at the end, and after the candles had been cleaned. These physical tests were used as a means of attempting to evaluate any changes in the filtering characteristics of the candles, in conjunction with bacteriological retention tests. Suspensions of the spores of ATCC No. 7064 *Bacillus cereus* and, later, cultures of *Serratia marsescens* were used both before and after the oil filtration test to evaluate the effectiveness of the filters in retaining all of the organisms. All filtrates were tested for sterility.

Physical Testing.—Table I lists the 18 different filters which were employed in the prolonged period filtration test. The first eleven filters listed were Selas[1] unglazed porcelain candles of the manufacturer's porosity designations 03, 02, 015, and 01. Seven of these, indicated by "H", were candles treated by the manufacturer with a silicone to render them hydrophobic. The next five filters listed were Mandler[2] diatomaceous earth candles of 18, 15, 11, 10, and 9 pounds p. s. i. bubbling pressure rating. Of the last two, the EK filter was a bacteria-retentive Seitz[3] asbestos pad filter and the sintered filter was a Pyrex[4] sintered glass 5/3 funnel filter.

Each of these filters was supplied with a reservoir for corn oil so that none of the filter surfaces, with the exception of the Selas 01A candle, became exposed to the air for more than a few minutes during the entire period of the test. The flow rate of the Selas 01A candle was too rapid to prevent the surface from becoming exposed. Oil filtrate was returned to the filters as much as possible throughout the test. Additional oil was added only when necessary to make up for loss due to spillage.

Findings.—The rate at which corn oil flowed through the filters is recorded in Table I together with the percentage of the final as compared with the initial flow rate. It will be noted that certain of the filters showed an increase in rate, or a rate nearly equal to that of the initial rate, at the end of the test period. The filters showing such an effect were all of the Mandler candles, with the exception of M11b which had been used previously in oil filtrations, the Selas 01A and the Seitz EK filter. At first consideration, these results might not appear to be significant. However, when compared with the markedly reduced flow rates of the other filters, the high flow rates would suggest the possibility that the filters had become less resistant to the flow of the oil and possibly less effective in retaining organisms. It would naturally be expected that the flow rate would become markedly reduced following prolonged filtration as a result of the gradual plugging of the pores.

The results of the bubbling pressure tests are also recorded in Table I. Those values marked with a question mark were so low that the gauge available

TABLE I.—EFFECTIVENESS OF FILTERS USING CORN OIL—PHYSICAL FACTORS

Filter	Total Vol. Filtered, ml.	Flow rate (ml./hour with approx. 5 mm. Hg) Initial	33 days	84 days	% of initial	Bubbling Pressure (p. s. i.) Initial CO_2-H_2O	Initial CO_2-Oil	Post-test CO_2-H_2O	Post-test CO_2-Oil	After Ignition Cleaning CO_2-H_2O	After Ignition Cleaning CO_2-Oil
Selas HA03[a]	743	10	6	3	30	1?	3	25	34	39	17
Selas HC03[b]	424	5	4	2	40	1?	3	25	38	39	17
+Selas HA02[a]	3,270	33	30	6	18	1?	11	19	23	30	12
Selas HC02[b]	102	6	2	0.25	4.1	1?	10	34	25	28	12
Selas HA015[a]	2,553	200	23	1.5	0.75	1?	13	7	14	12	5
Selas HC015[b]	673	35	7	1	2.85	1?	13	18	16	16	6
Selas HD015[b]	871	45	13	1	2.2	1?	10	14	16	15	6
+Selas 02A[b]	1,763	15	13	4.5	30	26	10	25	30	27	10
Selas 02C[b]	878	30	9	2	6.6	25	10	30	30	25	11
Selas 015F[b]	4,250	120	66	4.5	0.38	16	8	6	18	18	8
+Selas 01A[a]	238,280	1,500	1,750	1,750	116	9	2	4	6	8	2
+Mandler M18[c]	13,800	60	80	65	108	18	8	9	14	15[e]	8[e]
+Mandler M15[c]	22,660	92	117	75	81	15	3	8	10	13[e]	8[e]
Mandler M11b[c]	2,097	42	25	3	7.1	1?	1?	Broken	15	Broken	Broken
+Mandler M10[c]	46,800	205	243	225	110	11	3	6	10	10[e]	6[e]
+Mandler M9b[c]	18,882	65	87	75	115	1?	1?	4	5	8[e]	5[e]
+Seitz EK[d]	12,865	75	70	55	73	..	..	..	..	..	..
Pyrex Sintered 5/3[d]	Few drops only										

[a] App. 0.046 sq. ft. filter area.
[b] App. 0.023 sq. ft. filter area.
[c] App. 0.0341 sq. ft. filter area.
[d] App. 0.0276 sq. ft. filter area.
[e] After detergent cleaning.

[1] Selas Corporation of America, Philadelphia, Pa.
[2] Allen Filter Company, Toledo, Ohio.
[3] Hercules Filter Corp., Paterson 3, N. J.
[4] Corning Glass Works, Corning, N. Y.

TABLE II.—EFFECTIVENESS OF FILTERS USING CORN OIL—BACTERIOLOGICAL FACTORS[a]

Filter	Pretest: Volume Filtered, ml.—Saline Suspension A	Pretest: Volume Filtered, ml.—Filtrate Refiltered B	Pretest: Filtrate Tested (three 1 ml. inocula) B	Post Test: Volume Filtered, ml.—Corn Oil Suspension C	Post Test: Volume Filtered, ml.—Detergent Saline Washings D	Post Test: Results—Oil Filtrate C	Post Test: Results—Saline Filtrate D
Selas HA03	No filtrate obtained		..[b]	..	..	..	..
Selas HC03	No filtrate obtained		..	10	60	..	NG[d]
Selas HA02	100	None	2G[c]; 1NG	75	100	..	NG
				50	175	..	NG
Selas HCO2	100	None	NG	30	90	..	NG
Selas HA015	500	500	NG	100	200	NG	NG
Selas HC015	200	50	NG	60	200	..	NG
				60	160	..	NG
Selas HD015	200	50	NG	75	200	..	NG
				60	200	..	NG
Selas 02A	150	150	NG	..	..	..	..
Selas 02C	250	250	NG	..	..	..	..
Selas 015F	250	750	NG	60	200	..	NG
Selas 01A	100	900	1G; 2NG	100	200	NG	NG
				100	200	NG	NG
				60	200	..	NG
Mandler 18	450	150	NG	60	200	..	NG
Mandler 15	200	800	NG	75	200	..	NG
				60	200	..	NG
Mandler 11b	100	200	NG	..	..	..	..
Mandler 10	150	300	NG	100	200	NG	G
				100	200	..	NG
Mandler 9b	200	50	NG	..	..	..	..
Seitz EK	400	500	NG	..	..	..	..
Sintered 5/3	200	50	1G; 2NG	..	..	..	..

[a] Saline A containing approx. 900 spores *B. cereus* per ml. Corn Oil C containing approx. 600 spores *B. cereus* per ml.
[b] ... = No data.
[c] G = Growth.
[d] NG = No growth.

would not give an accurate reading. Following the filtration test period all of the candles showed an increase in the bubbling pressure when immersed in oil. All of the Selas hydrophobic candles also showed an increase when immersed in water. Four of the five candles which had shown a high flow rate at the end of the test period revealed a significant reduction in the bubbling pressure when immersed in water at the end of the test period. This finding suggests an increase in permeability of these filters.

After the candles had been cleaned, they were again tested for bubbling pressure. All of the unglazed porcelain candles were cleaned by ignition. Since the diatomaceous earth candles cannot be ignited, they were cleaned by soaking and filtration with detergents and then with petroleum ether and carbon tetrachloride. All of the unglazed porcelain candles gave bubbling pressure values essentially the same as when new, indicating that they were clean and that no permanent changes in the porosity had occurred. The unglazed porcelain hydrophobic candles gave what would be normal values for hydrophylic candles, indicating that the silicone had been removed. The diatomaceous earth candles gave less consistent results after cleaning, probably due to adsorbed films of detergent and/or oil remaining in the interstices of the candles. They were also found to be very susceptible to breakage when soaked with oil.

A sample of the last portions of the filtrates of the filters marked (+) in Table I was centrifuged and the sediment examined for fragments. None were observed.

As a check on possible changes in the oil itself, the specific gravity, viscosity, and refractive index of a sample of unused corn oil and of a composite of the final filtrates were determined. No difference was found between the two samples of oil (before and after filtration) in any of the determinations. It would, therefore, appear that no significant physical changes had occurred in the corn oil during the course of the study.

Bacteriological Testing.—Spores of the ATCC strain No. 7064 *Bacillus cereus* were used to test the bacteriological retention effectiveness of the filters. The Microbiological Section of the A. M. A. Laboratories recommends their use as the aerobic organism for sterility test studies. However, because of the large size of the spores of *B. cereus*, *Serratia marsescens* was also used in some of the final filtrations as an additional indication of the effectiveness of the filters in retaining microorganisms. As would be expected, some filters which retained all of the *B. cereus* spores did not retain all of the *S. marsescens*.

Suspensions of the spores of *B. cereus* in isotonic sodium chloride solution (hereafter designated as saline) were made according to the technique recommended by the A. M. A. Laboratories and diluted for use in filtration tests until they contained either approximately 900 or 600 spores per ml. as determined by the Millipore Filter technique.[5] The spores were heat shocked at 80° for thirty to sixty seconds in order to stimulate vegetation. Corn oil suspensions of the spores were made by shaking and rotating for one hour with 20 ml. of sterile corn oil in a tube containing 5-mm. glass beads or 2 Gm. of fine sand on which had been dried (in an incubator at 32°) a measured volume of the original saline suspension. This suspension in oil was also heat

[5] *Millipore Filters*, Millipore Filter Corp., Watertown, Mass.

TABLE III.—EFFECTIVENESS OF FILTERS USING CORN OIL—BACTERIOLOGICAL FACTORS—POST TEST

Filter	Volume Filtered, ml.		(A) Four 1 ml. Inocula of Filtrate into TSB	Volume ml. Saline Spore Suspension[a] Filtered	Inocula: (B) All of Filtrate into 2xTSB	Volume ml. Saline Suspension Filtered[b]	Inocula: (C) All of Filtrate into 2xTSB	Volume ml. Saline Suspension Filtered[b]	Inocula: (D) All of Filtrate into 2xTSB
	Saline[a] Spore Suspension	Filtrate Refiltered							
HA03	200	200	NG[c]	..[d]	..	..	..	..	..
HC03	30	..	NG	..	..	..	..	..	..
HA02	200	200	NG	..	..	..	..	..	..
HC02	40	..	NG	..	..	..	..	15	NG
HA015	200	200	NG	200	NG	200	G[e]	200	G
HC015	200	200	NG	200	NG	200	G	200	G
HD015	200	200	NG	..	..	..	..	..	..
02A	200	200	NG	..	..	..	..	..	..
02C	200	200	NG	..	..	..	..	..	..
015F	Disappeared	..	..	..	..	..	..	..	..
01A	200	200	NG	200	NG	200	G	200	G
M18	200	200	NG	200	NG	200	G	200	G
M15	200	200	NG	200	NG	200	NG	200	NG
M11b	Broken	..	..	..	..	..	..	..	..
M10	200	200	G	200	G	200	G	200	G
M9b	Broken	..	..	..	..	..	..	..	..
EK	Discarded	..	..	..	..	..	..	..	..
5/3	200	200	NG	..	..	..	..	..	..

[a] Approximately 600 spores *B. cereus* per ml.
[b] Approximately 600 spores *B. cereus* per ml. plus a 1:1000 dilution of a 24- to 48- hr. culture of *Serratia marsescens*.
[c] NG = No growth. [d] ... = No data. [e] G = Growth.

shocked at 80° and then diluted with additional sterile corn oil so that each ml. contained approximately 600 spores per ml., based on the spore content of the original saline suspension.

Prior to the filtration test, saline spore suspensions of *B. cereus* were filtered through the filters using the volumes indicated in column A, Table II. The filtrate was refiltered as indicated in column B to obtain the effect of larger volume filtration. The filtrate was then tested for the presence of spores by transferring three 1-ml. inocula from the last portions of the filtrates to test tubes of trypticase soya broth (TSB) and incubated at 32°.

Following the corn oil filtration test, a corn oil suspension of the spores of *B. cereus*, prepared as described above, was filtered through the filters. The volumes employed, in some cases with repeated trials, are recorded in Table II, column C. Sterile detergent saline (a 1:15 to 1:40 dilution of a 2% Tween® 80[6]-3% G2800[6] mixture or a 1/4 to 2 per cent Triton® X100[7] solution) was then filtered through the candles. This was followed by approximately an equal volume of sterile saline, the total combined volume of which is recorded in column D. Sterility tests were performed on four of the corn oil filtrates (C) thus obtained and on all of the saline filtrates (D). The methods used are described below. Detergent saline solutions were passed through the filters to determine whether or not spores could be carried through the filters, even though they may not have been carried through with the oil. Since the recovery of spores from oil is fraught with uncertainty, it was felt that any spores present in the filtrates could probably be more readily recovered from the saline washing filtrates than from the oil filtrates. Saline washing filtrates contaminated with spores and vegetative forms of *B. cereus* yielded positive findings upon subculturing after up to five days contact.

[6] Atlas Powder Co., Wilmington, Del.
[7] Rohm and Haas Co., Philadelphia, Pa.

Findings.—Growth was obtained from the filtrate of the Selas 01A and HA02 candles and from the sintered 5/3 filter as a result of the saline spore suspension filtration prior to the prolonged corn oil filtration test (filtrates B, Table II). The findings from the 01A candle could be expected because of the pore size of the filter. The possibility exists, however, that the positive tests with the HA02 candle and the 5/3 filter was due to faulty technique, since both of these filters gave no growth when sterility tests were performed on the filtrate following the prolonged corn oil filtration test, as noted below.

The results of the sterility tests on the filtrates from spore suspensions following the prolonged filtration test are recorded under filtrates C and D, Table II. Only the saline filtrate from the Mandler 10 candle gave evidence of the presence of organisms. Thus, it would appear that the other filters effectively retained all of the spores from the original oil suspension.

Sterility Testing.—Sterility tests were performed on the oil filtrates and upon the original corn oil spore suspension by the following methods.

Usually, one 1-ml. and two 2-ml. inocula were taken from each of the oil filtrates and three 1-ml. portions from the detergent-saline filtrates. From the original spore suspension in corn oil, 10 to 12 inocula of 0.5- to 2-ml. volumes were removed and tested.

Inocula from the oil samples were placed in TSB and shaken at intervals during incubation at 32°, in accordance with the U. S. P. sterility test directions for oleaginous liquids. In some cases fluid thioglycollate medium was also used. However, since an opalescence tended to develop in the fluid thioglycollate medium, the TSB medium was preferred. In some cases the oil inoculum in the TSB was centrifuged at about 1500 r. p. m. for one hour prior to incubation in an attempt to augment the transfer of the spores from the oil to the aqueous layer. In most cases growth was obtained when

TABLE IV.—EFFECTIVENESS OF FILTERS USING CORN OIL—BACTERIOLOGICAL FACTORS

Filter	Bubbling Pressure, p.s.i. CO_2-H_2O	Volume Saline[a] Filtered (ml.)	Inocula (A) 5 ml.	Inocula (A) Remainder	Volume Oil[b] Filtered (ml.)	Inocula (B) 5 ml. into 40 cc. TSB Shaken	Volume Oil[c] Filtered (ml.)	Inocula (C) 5 ml. into 50 ml. TSB Shaken	Cleaning	Volume Saline[a] Filtered (ml.)	Inocula (D) 5 ml.	Inocula (D) Remainder
					Immersed in Corn oil over-night							
Selas 02G (new)	23	200	NG[d]	NG	100	NG	100	NG	Ignition	200	NG	NG
Selas 015G (new)	18	200	NG	NG	100	NG	100	NG	Ignition	200	NG	G[e]
Selas HA02	28	200	NG	NG	ap. 20	NG	ap. 20	NG	Ignition	200	NG	NG
Selas HA015	12	200	G	G	100	NG	ap. 30	NG	Ignition	200	NG	NG
M 18	15	200	G	G	ap. 30	NG	50	NG	Boiled in 1/2% TRX 100 plus over-night in CCl_4	150	G	G
M 15	14	200	NG	NG	ap. 50	NG	ap. 20	G	Boiled in 1/2% TRX 100 plus over-night in CCl_4	200	G	G
Control		loopful	G		1 ml. 1 bead	G G	1 ml.	G		loopful	G	

[a] Approximately 600 spores *B. cereus* per ml. plus a 1:1000 dilution of a 24 to 48 hr. culture of *S. marsiscens*.
[b] Containing approximately 600 spores *B. cereus* per ml.
[c] Oil containing dispersion of *S. marsiscens* scraped from agar slant and shaken with five 5 mm. beads in sterile corn oil.
[d] NG = No Growth.
[e] G = Growth.

0.1 ml. of the original spore suspension in oil was tested by either of these methods.

Inocula of the oil samples were also emulsified by means of agitation with the transfer pipet in 10 ml. of a mixture of 2% Tween® 80 and 3% G2800. One ml. inocula of the coarse emulsion thus formed were transferred immediately to 10 ml. of TSB or fluid thioglycollate and were incubated at 32° with occasional shaking. Some of the tubes were also centrifuged at 1500 r. p. m. for one hour prior to incubation. In later studies a 1/4 to 1% Triton® X100 solution was used as the emulsifying agent. Transfer inocula were usually necessary to determine the presence of organisms after a period of incubation because of the cloudiness produced by the emulsion. There was no constancy with which organisms could be recovered, even from the original spore suspension in oil. When attempts were made to plate on trypticase soya agar an inocula of the emulsion prior to incubation, recovery of typical organisms was very poor.

Another method employed in an attempt to recover spores from oil samples was to disperse a 1 to 3 ml. inocula of the oil in 6 ml. of sterile polyethylene glycol 400. This mixture was usually centrifuged at 1500 r. p. m. for a period of one hour although a few were only shaken. One ml. inocula of either the sediment or of the shaken mixture was then transferred to 10 ml. of TSB and incubated at 32°. There was no constancy with which organisms were recovered from the original spore suspension in oil. When attempts were made to plate the mixture prior to incubation, recovery was infrequent.

As a control in the above sterility test procedures, a tiny grain of sand or a single 5-mm. bead, from those used to disperse spores in the first 20-ml. portion of oil, was transferred to 10 ml. of TSB. In every case, typical growth was obtained in 24 to 48 hours.

The saline filtrates were tested for sterility by transferring three 1-ml. inocula, each to tubes containing 10 ml. of TSB. The tubes were shaken and then incubated at 32°. The results obtained are recorded in the last column of Table II.

After the prolonged corn oil filtration test and subsequent to cleaning, an aqueous spore suspension of *B. cereus* was filtered through the candles and the sterility of the filtrate was determined in the same manner as was performed prior to the filtration test. The results obtained are recorded in the column marked A in Table III. Growth was obtained from only the filtrate

of the Mandler 10 candle. No growth was obtained prior to the filtration test from the aqueous filtrate from this candle.

Six of the filters were then subjected to a more stringent test in that all of the filtrate from 200 ml. of the saline spore suspension was incubated in 2x TSB. Again growth was obtained in the filtrate from only the Mandler 10 candle, as indicated in column B of Table III.

Since the spores of *B. cereus* are quite large, it was decided to add to the saline spore suspension sufficient volume of a 24- to 48-hour culture of *S. marsescens* to make a 1:1000 dilution. When this suspension was filtered through the same six filters, only the Mandler 15 candle did not show growth in the filtrate, as seen from column C, Table III. When repeated, only the Mandler 15 and the added Selas HCO2 candles did not show growth in the filtrate, as recorded in column D.

Modified Filtration Test.—Since *S. marsescens* had not been used with the filters prior to the prolonged corn oil filtration test, it was decided to conduct a modified corn oil filtration test. Two new filter candles were employed as well as four of those used previously. The procedure followed was essentially the same as for the prolonged corn oil filtration test, but with three modifications. First, the candles were immersed in the corn oil for a total period of approximately 24 hours; second, both spores of *B. cereus* and vegetative forms of *S. marsescens* were used to test the efficacy of the filters in retaining organisms; and third, a 5-ml. inoculum and the remainder of the filtrate from saline filtrations was incubated in 2x TSB. A single 5-ml. inoculum shaken in TSB from each oil filtrate was the basis for determining the sterility of the oil filtrates. The results are recorded in Table IV.

Findings.—Prior to immersion in oil, organisms were recovered from the saline filtrates from the Selas HA015 and the Mandler 18 candles. The latter candle was found to have a leak around the stem, however, and thus unfiltered material was passing into the filtrate. This was corrected for subsequent filtrations. Column B in Table IV shows that no *B. cereus* was recovered from corn oil filtrates from any of the candles. *S. marsescens* was recovered from the oil filtrate of the Mandler 15 candle (column C) when scrapings from an agar slant of the organism were dispersed in sterile corn oil and the oil filtered.

After cleaning, organisms were recovered from the remainder-inocula from the new Selas 015G candle and from both the 5-ml. inocula and the remainder-inocula from filtrates from both of the Mandler candles as recorded in column D. It should be pointed out that, despite detergent and solvent cleaning of the Mandler candles there was evidence of oil and detergent residue in the saline filtrates from these candles.

SUMMARY AND CONCLUSIONS

By means of alternating vacuum and gravity filtration, corn oil was passed continuously through a series of filters for a period of 84 days. The filters employed included Selas unglazed porcelain candles of 01, 015, 02, and 03 porosities, Mandler diatomaceous earth candles of 9, 10, 11, 15, and 18 p. s. i. bubbling-pressure test, a Seitz EK asbestos filter, and a Pyrex sintered 5/3 Buchner filter. Physical tests of filter effectiveness, i. e., flow rates and bubbling pressure determinations, were performed before and after the prolonged period of corn oil filtration. Filter effectiveness, as determined by sterility tests on filtrates from saline and from corn oil suspensions of *B. cereus* spores and *S. marsescens* were also performed before and after the prolonged period of corn oil filtration. Additional short period filtrations were performed to corroborate the results obtained from the prolonged filtration.

The findings show that there was an increased or relatively high flow rate under vacuum of corn oil through the Selas 01A candle, four out of the five Mandler candles, and the Seitz EK filter following prolonged corn oil filtration. This suggests that these filters became less resistant to the flow of corn oil and, possibly, may be less effective in retaining microorganisms. Four of the five candles which had shown the increased or relatively high flow rate also showed a decreased carbon dioxide-water bubbling pressure following the filtration test, whereas, all but one of the other candles either showed an increase or remained about the same. This also suggests an increase in permeability of the above mentioned filters. The increased fragility noted when the Mandler candles were soaked with oil and the great difficulty in effectively removing all of the oil from the candles further reduces the desirability of using these candles for the filtration of corn oil.

The results from the sterility tests on the filtrates suggest that the Mandler candles are less effective in retaining all *B. cereus* spores and *S. marsescens* from suspensions in both corn oil and saline when the candles are used for the filtration of corn oil. The effectiveness of Selas 015 candles for the filtration of corn oil to obtain a sterile filtrate is not dependable with organisms such as *S. marsescens*.

The findings from this preliminary study suggest that certain filters are less effective following corn oil filtration. Further investigation is indicated. It is hoped that others will undertake similar studies so that ample proof may be made available as to the effectiveness of the marketed bacteria-excluding filters in retaining all microorganisms which may be present in contaminated oils.

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Toxicity and Chemistry of 11-Desmethoxyreserpine (Raunormine[1]) (3,4,5-Trimethoxybenzoic Acid Ester of Methyl-11-desmethoxyreserpate), an Alkaloid from *Rauwolfia canescens* I*

By JOS. W. E. HARRISSON, ELIAS W. PACKMAN, ERICK SMITH†, NORMAN HOSANSKY†, and RALPH SALKIN†

Rauwolfia canescens yields another alkaloid which possesses sedative and strong hypotensive activity. This newly isolated ester alkaloid has been termed 11-desmethoxyreserpine. Unlike reserpine, this alkaloid does not yield reserpic acid upon hydrolysis. The acid resulting as a hydrolysis product of 11-desmethoxyreserpine has been termed raunormic acid, whose physical properties differ markedly from those of reserpic acid. Acute oral, intraperitoneal, and chronic oral toxicity studies indicate that 11-desmethoxyreserpine exhibits in the test animal a favorable therapeutic index, thus assuring relative safety, warranting further pharmacologic and clinical studies.

At least two sedative principles have previously been isolated from Rauwolfia species: reserpine from *Rauwolfia serpentina* (1) and the other species of Rauwolfia (2), and rescinnamine from *Rauwolfia serpentina* (3). Both compounds are esters of methyl reserpate; reserpine is the 3,4,5-trimethoxybenzoic acid ester and rescinnamine is the 3,4,5-trimethoxycinnamic acid ester.

Recently there has been isolated a new ester alkaloid from *Rauwolfia canescens* which has been termed 11-desmethoxyreserpine.

During the course of study on this alkaloid Stoll and Hofmann (4) of Sandoz Inc. reported the isolation of canescine and suggested that a methoxy group in the 11 position was not necessary for hypotensive activity; Schlittler, *et al.* (5), of Ciba Pharmaceutical Laboratories reported deserpidine and suggested a structure; MacPhillamy, *et al.* (6), of Ciba announced the conversion of their deserpidine to a-yohimbine; Slater, *et al.* (7), of Lilly Research Laboratories announced the separation of recanescine, proposed a structure, reported upon ptosis, sedation, and hypotonic activity and also concluded that the methoxy of reserpine at the 11 position was not necessary for reserpine activity; S. B. Penick & Company Research Laboratories (8) announced the separation of raunormine a new hypotensive alkaloid from Rauwolfia. Since this manuscript was prepared Schneider, *et al.* (9), of Ciba have published additional pharmacologic information.

A comparison of the overlapping physical and chemical properties of these substances shows the identity of the alkaloid we are reporting and that of the others to be 11-desmethoxyreserpine. We are extending the data on the degradation products and reporting upon the toxicity.

This alkaloid possesses sedative and strong hypotensive activity (10). Comparison of the physical and chemical properties with reserpine and rauwolscine (11) indicates that the compounds are not identical. Hydrolysis, as described below, yields an acid (raunormic acid) differing from reserpic acid in both ultraviolet (Fig. 1) and infrared (Fig. 2 a and b) absorption. On the basis of these data desmethoxyreserpine is postulated as the 3,4,5-trimethoxybenzoic acid ester of methyl-11-desmethoxyreserpate (Fig. 3). The similarity of the solubility of 11-desmethoxyreserpine and reserpine accounts for the difficulty encountered in isolation of pure reserpine from *R. canescens*. The base desmethoxyreserpine was obtained from the mother liquors after the crystallization of reserpine. When crystallized from ethyl acetate it separates as shining white rods containing one mole of solvent.

PHYSICAL PROPERTIES OF THE SOLVENT-FREE BASE

Melting point 224–226°; $[\alpha]_D$ −136° (c = 1, chloroform) and −162° (c = 0.5, pyridine); λ max. 218 mμ (log ε 4.76) and 271–272 mμ (log ε 4.25); λ min. 243–244 mμ (log ε 3.79) (in ethyl alcohol).

Anal.—Calcd. for $C_{32}H_{38}O_9N_2$: 578.6; C, 66.42; H, 6.62; N, 4.84, OCH_3 (5), 26.82. Found: C, 66.45; H, 7.00; N, 5.06; OCH_3, 26.72; mol. wt. by anhydrous titration in dioxane, 581.

The alkaloid forms well-defined salts, for example the nitrate: m. p. 244–246°.

* Received April 28, 1955, from LaWall and Harrisson Research Laboratories, Philadelphia, Pa. Presented to the Scientific Section, A. Ph. A., Miami Beach meeting, May, 1955.

† S. B. Penick & Co., Research Laboratory, Jersey City, N. J.

[1] Raunormine is trademark of S. B. Penick & Co. for their 11-desmethoxyreserpine which was used in this study.

The isolation of desmethoxyreserpine (Raunormine) used in this study and the determination of its physical and chemical properties were made by the Research Laboratory of S. B. Penick and Company. The reserpine was also supplied by S. B. Penick & Company.

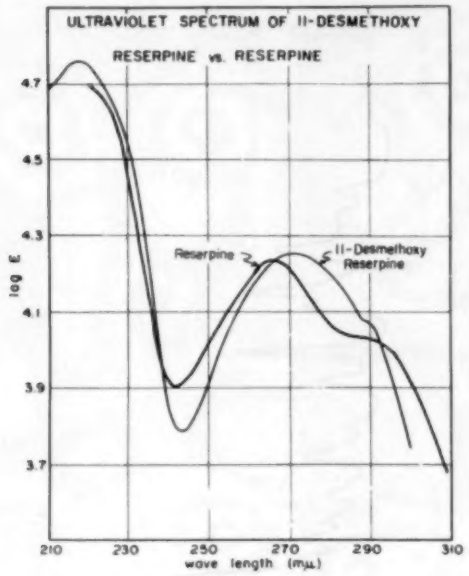


Figure 1

Anal.—Calcd. for $C_{22}H_{32}O_9N_2 \cdot HNO_3$: C, 59.90; H, 6.13; N, 6.55. Found: C, 59.86; H, 6.32; N, 6.57.

The oxalate: m. p. 234–236°.

Anal.—Calcd. for $C_{22}H_{32}O_9N_2 \cdot C_2H_2O_4 \cdot {}^1/_2H_2O$: C, 60.26; H, 6.10; N, 4.13. Found: C, 60.44; H, 6.47; N, 4.44.

On alkaline hydrolysis with sodium hydroxide in dilute methanol, raunormine gives a theoretical yield of 3,4,5-trimethoxybenzoic acid is obtained, m. p. 169–171° with no mixed melting point depression with authentic acid.

Anal.—Calcd. for $C_{10}H_{12}O_5$: C, 56.60; H, 5.70; OCH_3 (3), 43.87. Found, C, 56.80; H, 5.47; OCH_3, 43.96.

From the mother liquors raunormic acid was isolated as its hydrochloride and purified by crystallization from absolute ethanol; m. p. 248–250°, $[\alpha]_D$ −80.5° (c = 0.5, alcohol). However, it proved on analysis to contain water of crystallization which could not be removed by drying.

Anal.—Calcd. for $C_{21}H_{30}O_4N_2 \cdot HCl \cdot 1.5\,H_2O$: mol. wt., 433.9: C, 58.12; H, 6.97; N, 6.46; Cl, 8.17; OCH_3, 7.15. Found: C, 57.91; H, 6.89; N, 6.31; Cl, 8.01; OCH_3, 7.03. Similar behavior was noticed with reserpic acid hydrochloride (12).

The free acid prepared from the hydrochloride with silver carbonate and crystallized from alcohol melts at 247–248°; $[\alpha_D]$ −93° (c = 0.2 alcohol).

Anal.—Calcd. for $C_{21}H_{28}O_4N_2 \cdot H_2O$, mol. wt. 388.45; C, 64.93; H, 7.27; N, 7.21. Found: C, 65.32; H, 7.47; N, 6.82.

The infrared spectrum (Nujol® mull) (Fig. 4a and 4b) is similar to that of reserpine, but it does not show the strong reserpine band at 6.15 μ which is the most useful point for comparison. The infrared absorption spectrum of raunormic acid hydrochloride differs markedly from that of reserpic acid hydrochloride in that the former lacks the absorption bands for the aromatic OCH_3 at 7.94 and 9.52μ. Furthermore, the bands found in reserpic acid at 6.25, 6.35, 11.5, and 12.2μ, indicative of a 1,2,4-substituted benzene ring, are missing in raunormic acid.

Preliminary pharmacological tests show it to have sedative as well as hypotensive activity comparable to reserpine (10).

ACUTE TOXICITY

The acute toxicity to rodents and dogs is relatively low. Intraperitoneal toxicity data obtained with male Webster Swiss albino mice,[1] indicate that the death rate increases during successive days of observation (Table I). We have observed that within fifteen minutes of injection, the animals fall into a comatose state and remain in this sleeping stage for several days, therefore, the deaths observed on successive days may be due partly to starvation and dehydration. For this reason, we have chosen 48 hours as the elapsed time upon which to base our LD_{50} calculations.

The alkaloid was dissolved in a 1:1:2 mixture of ethyl alcohol, propylene glycol, and water for both intraperitoneal and oral administration to rodents.

The LD_{50} in mice after intraperitoneal injection is approximately 60 mg./Kg. Following similar administration, the LD_{100} is more than twice this amount, 130 mg./Kg. These results (Table I) are in sharp contrast to the relatively high dosages producing death in mice following oral administration (Table II).

TABLE I.—ACUTE INTRAPERITONEAL TOXICITY OF 11-DESMETHOXYRESERPINE IN MICE

Log Dose	Dose mg./Kg.	Deaths 24 Hrs.	48 Hrs.	72 Hrs.
1.4	25.1	2/20	2/20	2/20
1.6	39.8	3/20	7/20	8/20
1.8	63.1	0/20	14/20	14/20
1.9	79.4	10/20	16/20	16/20
2.0	100.0	14/20	17/20	19/20
2.1	125.9	16/20	18/20	18/20

LD_{50}, 24 hrs. = 67.7 mg./Kg. ± 12.8 mg.
LD_{50}, 48 hrs. = 58.0 mg./Kg. ± 8.5 mg.
LD_{50}, 72 hrs. = 55.3 mg./Kg. ± 8.6 mg.

TABLE II.—ACUTE ORAL TOXICITY OF 11-DESMETHOXYRESERPINE IN MICE

Log Dose	Dose mg./Kg.	Deaths 24 Hrs.	48 Hrs.	72 Hrs.
2.4	251.2	0/10	2/10	2/10
2.5	316.2	0/10	4/10	4/10
2.6	398.1	0/10	2/10	5/10
2.7	501.2	0/10	6/10	7/10
2.87	750.0	Not observed	8/10	9/10

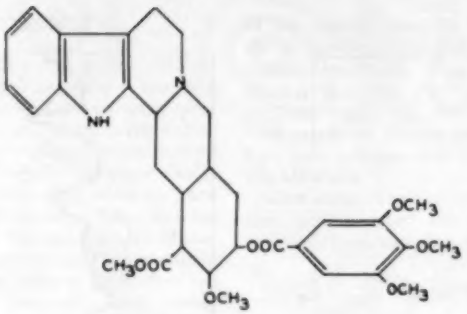
LD_{50}, 24 hrs.
LD_{50}, 48 hrs. = 512 mg./Kg. ± 83.4 mg.
LD_{50}, 72 hrs. = 316 mg./Kg. ± 30.1 mg.

After oral administration no deaths were observed during the first 24-hour observation period with dosages as large as 500 mg./Kg., although the mice were in a comatose state. Some deaths were observed at the 48-hour observation, and the LD_{50} was calculated to be approximately 513 mg./Kg., while the LD_{100} was not reached even at 750 mg./Kg. (Table II).

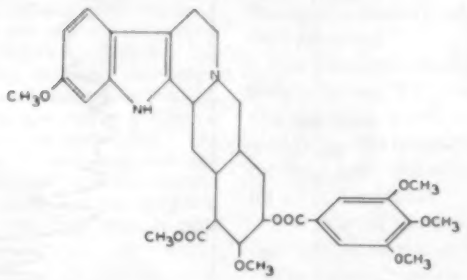
[1] Taconic Farms, Germantown, N. Y.

Fig. 2a.—11-Desmethoxy Reserpine

Fig. 2b.—Reserpine



11-DESMETHOXY RESERPINE



RESERPINE

Figure 3.

TABLE III. EFFECT OF FEEDING 11-DESMETHOXYRESERPINE OR RESERPINE IN THE DIET OF THE RAT.

Survivals, Average Body Weight, Food and Water Intake in Grams on Weekly Basis

		0 Week	1st Week				2nd Week				3rd Week				4th Week			
		Weight	% in Diet	Survivals	Wgt.	Food	Water	% in Diet	Survivals	Wgt.	Food	Water	% in Diet	Survivals	Wgt.	Food	Water	% in Diet	Survivals	Wgt.	Food	Water
					(in grams)					(in grams)					(in grams)					(in grams)		
A Level - 11-Desmethoxy Reserpine (Low Level)	Females	65.6	0.0005	15/15	71.8	44.2	97.1	0.0005	14/15	75.5	73.2	151.7	0.0005	13/15	97.5	110.0	145.0	0.0005	13/15	104.6	119.5	234.6
	Males	71.4		15/15	72.8	61.4	129.2		9/15	77.2	116.5	150.0		6/15	92.8	111.6	124.6		6/15	102.7	112.1	230.1
B Level - 11-Desmethoxy Reserpine (High Level)	Females	68.4	0.001	15/15	73.4	54.5	96.5	0.001	6/15	63.4	56.5	91.5	0.001	3/15	66.6	79.0	84.0	0.001	3/15	62.3	62.0	92.6
	Males	77.8		12/13	63.0	63.1	97.8		4/15	57.5	47.0	107.0		2/13	60.0	80.5	99.0		2/13	63.5	67.0	126.0
C Level - Reserpine	Females	67.6	0.0005	14/15	63.2	42.2	93.5	0.0005	4/15	51.0	66.0	99.2	0.0005	0/15								
	Males	69.2		13/15	64.2	37.4	78.4		5/15	62.8	79.2	104.3		3/15	58.3	71.5	82.6		0/15			
BL - Control	Females	68.1		15/15	73.8	84.6	117.6		15/15	103.6	123.8	257.3		15/15	137.0	146.0	228.6		15/15	123.6	150.0	257.2
	Males	74.7		15/15	82.6	77.7	104.2		14/15	101.0	115.5	229.1		14/15	130.8	149.2	218.9		14/15	127.2	149.5	226.2

In addition to recording mortality at the various dosage levels the following observations were made:

Upon intraperitoneal injection, the mice fell into a deep sleep almost immediately and exhibited labored breathing. Diarrhea was evident approximately three hours after injection. The surviving mice, when observed 72 hours after injection, had their eyes closed and were somewhat sedated.

Similar side effects were observed after oral administration; however, the onset of these symptoms was only evident several hours after administration.

In acute studies, 10-Kg. dogs have survived after single oral doses of up to 300 mg. of dry alkaloid. These animals became very quiet, and appeared to have lost interest in their surroundings. Miosis was observed three hours after administration. Labored breathing, diarrhea, and profuse salivation were also evident. The appetite of the animals was lost for approximately three days; however, eating habits returned to normal on the sixth day. No hematologic changes occurred during the period of observation. Dogs receiving 600 mg. of dry alkaloid orally survived only for 24 hours.

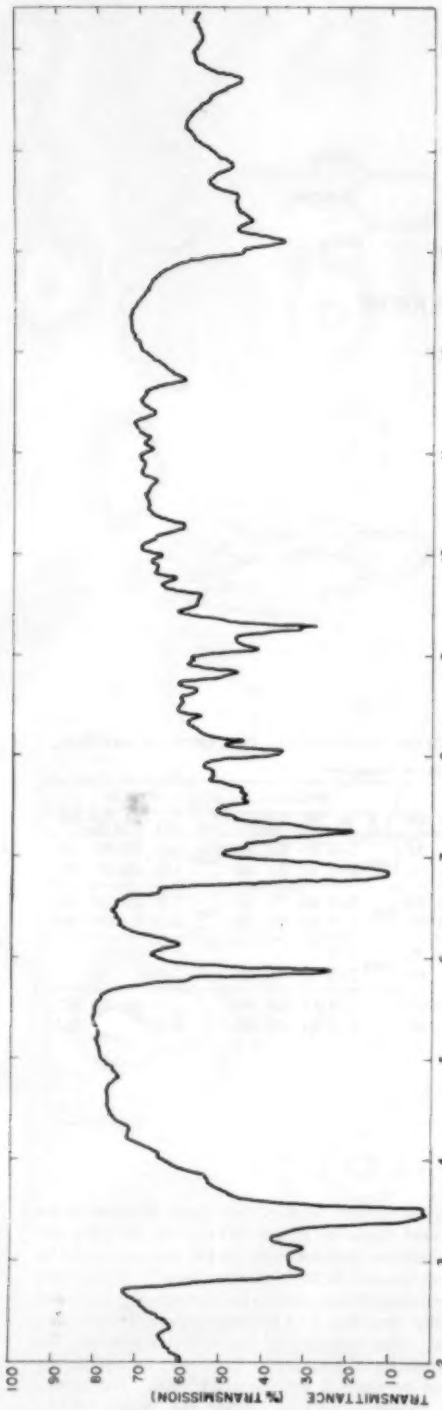


Fig. 4a.—Raunormic Acic

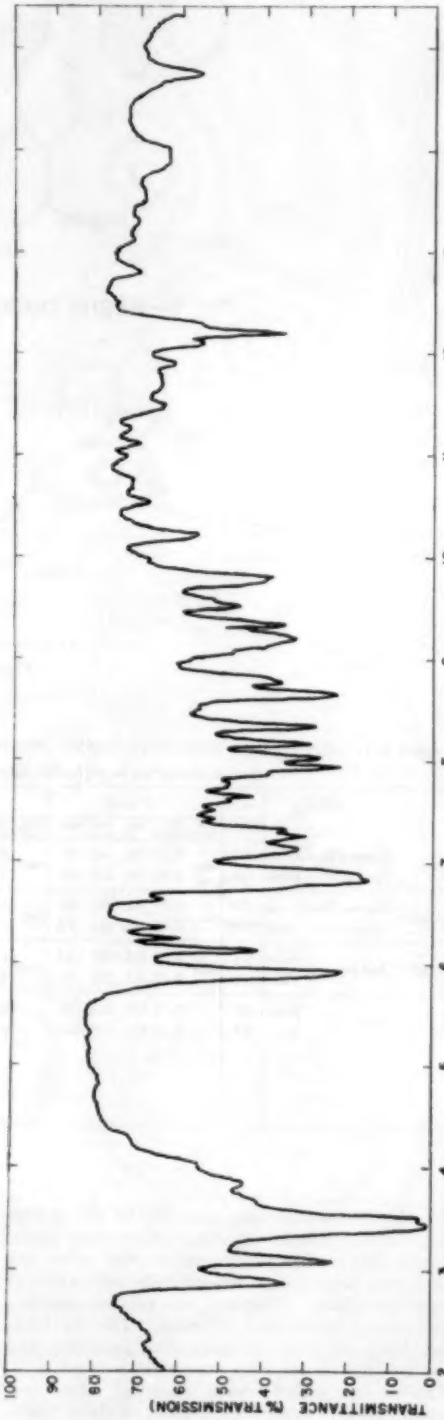


Fig. 4b.—Reserpic Acid

SUB-ACUTE TOXICITY

Oral administration of 20 mg./Kg. dry alkaloid was made to 10-Kg. mongrel dogs for a four-day period; three of four dogs died seven days after the animals were placed on test, or three days after the last oral administration. These animals reacted in a manner similar to the dogs in the acute study. Due to the marked sedative effect they stopped eating on the second day of the test. The surviving dog, who received a total of 800 mg. over a four-day period showed no hematologic change, but remained in a stage of tranquility for thirteen days after the last oral administration. Appetite and eating habits of this dog were approximately normal after administration was discontinued.

In a dosage of 10 mg./Kg. per day orally it was tolerated by three 10-Kg. mongrel dogs for five days. Miosis and increased defecation was evident several days after the first administration. This was followed by labored breathing, increased salivation, then a state of tranquility, and finally sedation. A 10-mm. drop in blood pressure as well as a decrease in heart rate was observed on the second day. The appetite and eating habits of these dogs although below normal was sufficient to maintain the animals. One of the dogs died two days after the drug was discontinued. The other two dogs were in good health after ten days.

CHRONIC TOXICITY

One-hundred-twenty litter-mate rats[3] twenty-eight days of age were divided into four test groups. 11-Desmethoxyreserpine was incorporated in the diet of two groups, reserpine in the diet of one group, and the other group was maintained as a control group which received only the base diet, Rockland Rat Meal. All groups except one contained an equal number of female and male animals. Each animal was individually housed in a suspended open mesh cage in air-conditioned quarters. The respective diet and water were supplied *ad libitum*.

The alkaloids were ground in a small ball mill with a quantity of sucrose before gradual dilution with the basal diet. During the first week that the animals were on the test, 0.0005% (low level) and 0.001% (high level) of 11-desmethoxyreserpine and 0.0005% of reserpine were incorporated in the respective diets. Thereafter the amount of alkaloid in each of the diets was increased 50%. At this higher level of inclusion the dosage intake corresponded to 1 mg. and 2 mg./Kg. of test animal per day of the 11-desmethoxyreserpine and 1 mg./Kg. of the reserpine. All levels of inclusion depressed the consumption of food and thus likewise the gain in weight as compared with the control animals. Among those animals receiving 1 mg./Kg. of desmethoxyreserpine, 80% of the males and 40% of the females survived the four-week test period. On an equivalent intake of reserpine, none of the animals survived. When 2 mg./Kg. of 11-desmethoxyreserpine was administered approximately 20% of males and females survived. The lower food intake of all test animals may have been due to a taste aversion and to the tranquilizing effect of the alkaloids.

The mean values at the several weekly observation periods show the effect upon weight gain, survival, food and water intake (Table III).

[3] Sprague Dawley Strain.

CONCLUSIONS

The physical properties of 11-desmethoxyreserpine, a new ester alkaloid isolated from *Rauwolfia canescens*, and of raunormine acid have been presented.

The intraperitoneal LD_{50} in mice is approximately 60 mg./Kg., while after oral administration the LD_{50} is 500 mg./Kg. Dogs have tolerated single oral doses of 300 mg. while others have survived after oral administration of 10 mg./Kg. for a five-day period.

The ratio of the acute peritoneal to the acute oral dose in mice is approximately 1:8.

The approximate chronic LD_{50} for 11-desmethoxyreserpine when administered to the white rat in the diet for a period of thirty days is 1 mg./Kg. of test animal.

Preliminary studies indicate that the acute, to subacute and chronic toxicity of 11-desmethoxyreserpine is comparable to reserpine.

This alkaloid has been variously named: canescine, deserpidine, and recanescine.

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A Study of Antacids II*

Comparative Evaluation by Various Methods

BY ROGER E. BOOTH and JACK K. DALE

Nine *in vitro* methods commonly used to evaluate antacids were compared on several types of antacids. No direct correlation could be made to reduce all the methods to a common denominator. The effects on results due to changes in temperature and stirring rate were investigated. These changes may be characteristic of the antacid as well as the method. Reproducibility studies show the need for several runs to obtain a valid evaluation. The data presented substantiate the thesis that promotional literature for antacid products should be based on a standardized method. A comparison of various antacids by a particular method gives useful information. However, further work needs to be done to find a standardized method suitable for all types of antacids.

IN ORDER to evaluate new antacid formulations and thereby produce more effective ones, various methods have been proposed. The criteria for choosing any particular method may vary with the thinking and purposes of the investigator. In a recent paper (1), many formulas were evaluated by an *in vitro* method which seemed to be closely related to actual clinical data (2). In addition, this method gave other useful information such as rapidity of action, maximum *p*H obtained, duration of action to certain *p*H levels and mechanical ease of operation. Further considerations in choosing a general method might include studies on necessary agitation rates and temperature conditions, apparent emptying time of the stomach, variation in acid secretion and gastric juice composition, methods of speeding reaction times, and generally available equipment. No standardized method has been generally accepted as yet. The official tests are used as required but do not give sufficient evaluating data.

Particularly while studying promotional literature, it occurred to us that each product was acclaimed the best based on a certain evaluating method. The methods used, although similar in some respects, could not be directly correlated. This led to the study of typical products by all the methods available at the time. In addition, a study of some of the conditions employed in two representative type methods showed the variation in results which may be expected with variations in temperature and stirring rates.

EXPERIMENTAL

Nine methods were compared in this study. These methods are schematically described in Table I. They can be grouped into two main divisions: those which periodically add simulated gastric mixtures and those which periodically add and *empty* similar mixtures. For further study, a type method considered adequate in each of these groups was evaluated as to temperature, rate of stirring and reproducibility. The antacids studied consisted of outstanding ones of various types. These were tablets of (*a*) magnesium trisilicate plus aluminum hydroxide, (*b*) magnesium carbonate plus magnesium trisilicate plus calcium carbonate, and (*c*) magnesium carbonate plus magnesium oxide plus calcium carbonate. They were selected as representative of a slow reacting type, a combination of slow and fast reacting type, and a fast reacting type respectively.

Figures 1 and 2 give typical titration curves by eight of the methods on a single lot of the particular antacid. To avoid confusion only the peak values have been plotted in all the methods. Because of the extended time axis required Method IX is plotted separately in Fig. 3 and 4. The wide range of apparent activity which results from the evaluation of any particular antacid by six of the methods is clearly shown in Table II.

Method I was chosen as representative of the "addition" group; in the "addition-emptying" group, Method IV was chosen. These methods were run in parallel at temperatures of 25° and 37°. Typical curves are given in Fig. 5–10.

The rate of stirring was studied at three speeds. Speed (1x) corresponded to the center line of a cone driven stirrer. Speed (2x) was approximately twice speed (1x); speed ($^1/_2$x) was approximately one-half speed (1x). The exact rate of stirring (r. p. m.) is considered unessential for comparative purposes when run in the same laboratory with the same equipment. Figures 11 through 14 give typical curves of parallel runs varying only the stirring rates.

Reproducibility of Methods I and IV is given in Fig. 15 and 16. The curve drawn is the mean value at each point. Maxima and minima values are indicated by the vertical lines.

DISCUSSION OF RESULTS

The data obtained in this study emphasize the need for a widely accepted standard evaluating method for antacids. This is particularly true when such curves are considered indicative of clinical

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Presented to the Scientific Section, A. PH. A., Miami Beach meeting, May 1955.

TABLE I.—ANTACID EVALUATING METHODS

Method	I	II	III	IV	V	VI	VII	VIII	IX
Lit. Reference	(2)	(3)	(4)	(5)	(6)	(4, 7)	(8)	(9)	(10)
Procedure									
To	50 ml.	25 ml.	150 ml.	150 ml.	100 ml.	150 ml.	100 ml.	203 ml.	100 ml.
Test fluid	0.1 *N* HCl	0.1 *N* HCl	0.05 *N* HCl 0.2% pepsin	0.05 *N* HCl 0.2% NaCl 0.32% pepsin	0.1 *N* HCl	0.05 *N* HCl 0.2% pepsin	0.05 *N* HCl 0.15% pepsin 0.15% peptone 0.15% NaCl	3 ml. 1 *N* HCl 200 H_2O	H_2O
At	37.5° C.	25° C.	38° C.	37.5° C.	37.5° C.	38° C.	38° C.	R. T.	37° C.
Is added indicated amt. of antacid to be tested	Desired amt.[a]	8 ml. or recom. max. dose[a]	1 Gm. or 1 tablet[a]	Desired amount[a]	Two equiv. doses[b]	Desired amount[a,c]	Neut. value[d] 20%[a]	1 Gm.[a]	1 tablet[a]
The *p*H is determined at (with stirring)	0, 1, 3, 5, 10	0, 1/2, 5, 10, 15, 20, 25, 30	0, 1, 3, 5, 10, 15, 20, 30	0, 1/2, 2, 4, 6, 8, 10	0, 1, 3, 5, 10	0, 1/2, 2, 4, 6, 8, 10	0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 60	5, 10, 15, 20, 25, 30	10, 11
At end of each	10 min.	30 min.	..	10 min.	10 min.	10 min.	..	10 min.	10 min.
Take out (fluid)	None	None	None	25 ml.	20 ml.	20 ml.	None	None	None
and add	2 ml. of 1 *N* HCl	25 ml. of 0.1 *N* HCl	None ..	25 ml. of Test fluid	20 ml. of 0.1 *N* HCl	20 ml. of Test fluid	None ..	1 ml. of 1 *N* HCl	10 ml. of 0.1 *N* HlC
Read *p*H again after each	10 min.	30 min.	..	2, 4, 6, 8, 10 min.	10 min.	2, 4, 6, 8, 10 min.	..	10 min.	10 min.

[a] Dosage tested in this report was 1 tablet equivalent to 1 Gm. total active ingredients.
[b] Equivalent dose is amount of antacid which when added to 100 ml. 0.1 *N* HCl at 37.5°C. gives a *p*H of 3.5 after 15 minutes.
[c] Hobart, Noble, and Grote (4) tested 2-Gm. doses. Armstrong and Martin (7) tested official doses.
[d] "Neutralizing value" is amount of antacid required to neutralize 100 ml. of 0.05 *N* HCl at 38° C. using bromphenol blue as indicator.

TABLE II.—COMPARATIVE EVALUATION DATA BY SIX METHODS

Method	Product (*a*)[a]						Product (*b*)[b]					
	I	II	III	IV	V	VI	I	II	III	IV	V	VI
Maximum *p*H attained	3.7	4.0	3.0	2.1	3.7	2.4	7.4	7.7	6.9	6.2	6.9	6.2
Minutes to reach *p*H 3.5 or above	5	5	*a*	*a*	5	*a*	1	0.5	1	0.5	1	0.5
Hours above *p*H												
4	0	0.5	0	0	0	0	1	>3	>0.5	0.7	0.5	1
3	1	>3	0	0	0	0	1	>3	>0.5	0.7	0.5	1
2	1.3	>3	0.5	0.9	1.1	1.1	1	>3	>0.5	1	0.7	1.3
Volume 0.1 *N* HCl equivalent neutralized to *p*H												
4	0	25	0	0	0	0	150	>100	>150	<113	<140	<125
3	150	>150	150	0	200	0	150	>100	>150	<125	<140	<135
2	190	>150	>150	125	220	135	150	>100	>150	<137	<160	<145

[a] Product (*a*) contains aluminum hydroxide dried gel powder, 0.65 Gm. plus magnesium trisilicate, 0.32 Gm.
[b] Product (*b*) contains calcium carbonate, 0.78 Gm. plus magnesium carbonate, 0.07 Gm. plus magnesium trisilicate, 0.13 Gm.

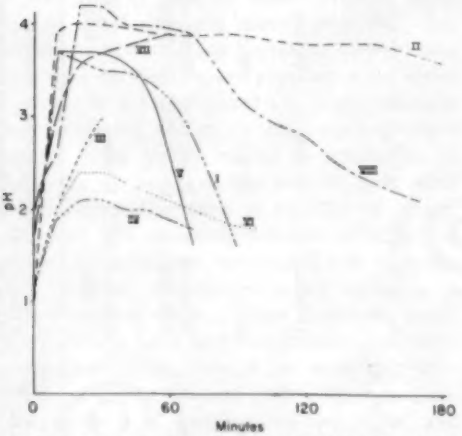

Fig. 1.—Methods I–VIII on Product (*a*) (aluminum hydroxide dried gel powder, 0.65 Gm., plus magnesium trisilicate, 0.32 Gm.)

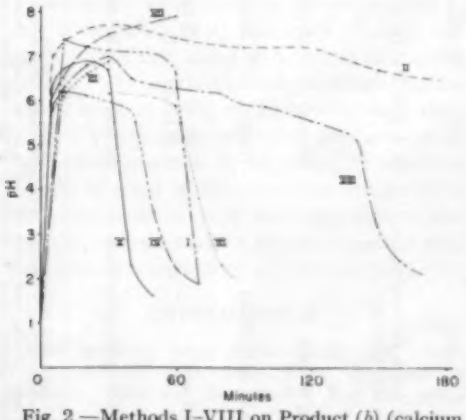

Fig. 2.—Methods I–VIII on Product (*b*) (calcium carbonate, 0.78 Gm., plus magnesium carbonate, 0.07 Gm., plus magnesium trisilicate, 0.13 Gm.)

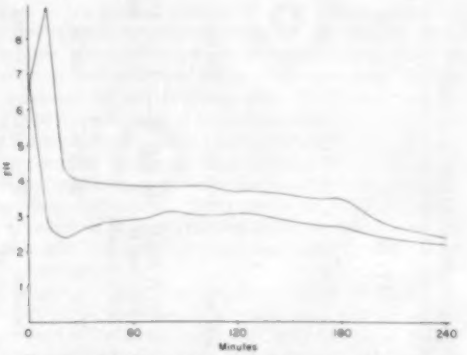

Fig. 3.—Method IX on Product (*a*) (aluminum hydroxide gel dried powder, 0.65 Gm., plus magnesium trisilicate, 0.32 Gm.)

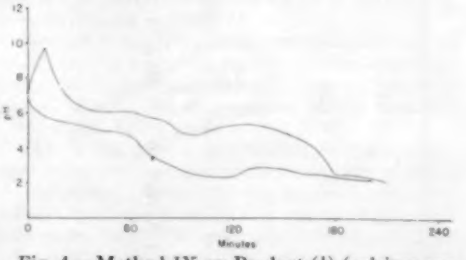

Fig. 4.—Method IX on Product (*b*) (calcium carbonate, 0.78 Gm., plus magnesium carbonate, 0.07 Gm., plus magnesium trisilicate, 0.13 Gm.)

effectiveness. Reference to Fig. 1 and 2 and Table II shows strikingly the variety of impressions that may be obtained about any particular antacid if evaluated by any certain method. For example antacid (*a*) would be considered poor if only Method IV were reported and very satisfactory if only Method II were reported. At the same time Method II places antacid (*b*) slightly above the usual desirable *p*H range while Method VIII gives an

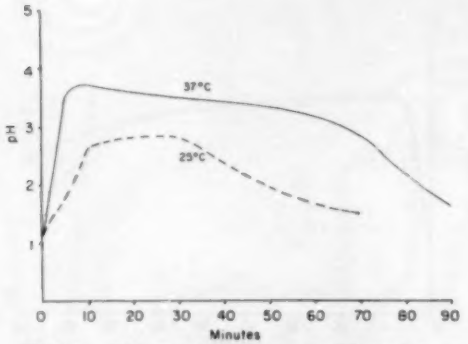

Fig. 5.—Temperature effect by Method I on Product (*a*) (aluminum hydroxide gel dried powder, 0.65 Gm., plus magnesium trisilicate, 0.32 Gm.)

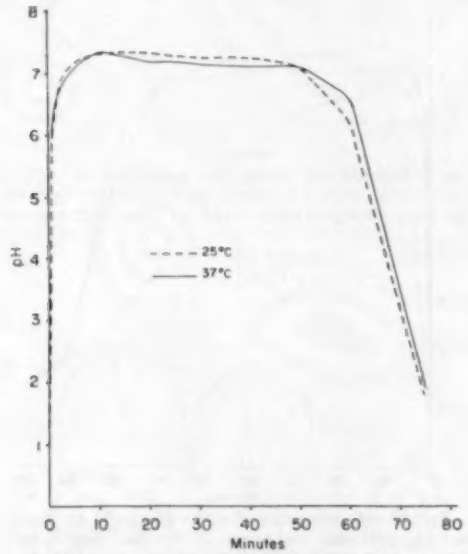

Fig. 6.—Temperature effect by Method I on Product (*b*) (calcium carbonate, 0.78 Gm., plus magnesium carbonate, 0.07 Gm., plus magnesium trisilicate, 0.13 Gm.)

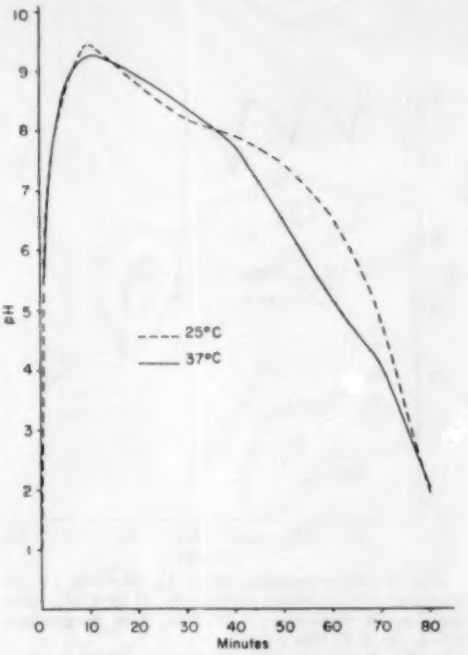

Fig. 7.—Temperature effect by Method I on Product (*c*) (calcium carbonate, 0.78 Gm., plus magnesium carbonate, 0.13 Gm., plus magnesium oxide, 0.07 Gm.)

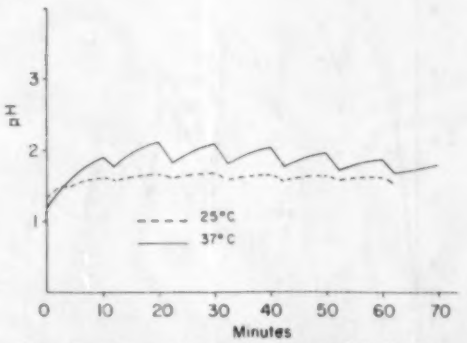

Fig. 8.—Temperature effect by Method IV on Product (*a*) (aluminum hydroxide gel powder, 0.65 Gm , plus magnesium trisilicate, 0.32 Gm.)

exceptionally favorable picture. Numerous other examples may be taken from the data given. The evaluation of any antacid formula by available methods is valid only on a comparative basis, for which there is no recognized standard.

In addition to the *choice* of a method, the *conditions* employed in the use of the method are critical in certain cases. Consider the type methods studied.

Rate of Stirring.—Both Methods I and IV gave considerably different curves when the rate of stirring was altered (cf. Fig. 11–14). It is assumed that the geometry of the stirring apparatus is as critical as the actual rate of stirring, hence the same equipment was always used. Each of the products gave elevated curves as the stirring rate increased. Product (*a*) by Method I may be an exception since the curves ran together for seventy minutes. After this, the curve of the highest speed dropped off more rapidly than the others. This may indicate that the capacity of the slowly reacting aluminum hydroxide was depleted more rapidly at higher speeds.

Temperature.—Temperature studies illustrate the differences in specific type products (cf. Fig. 5–10). Product (*a*) showed wide variations when run at different temperatures—the higher temperature giving an elevated curve. However, the more rapid acting products (*b*) and (*c*) were largely unaffected by this temperature change. Again, a possible exception,

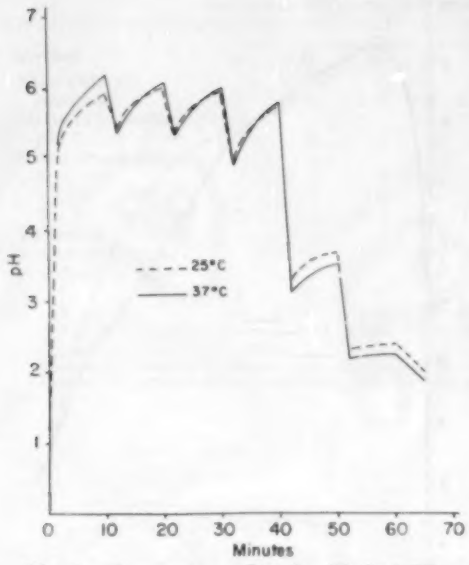

Fig. 9.—Temperature effect by Method IV on Product (*b*) (calcium carbonate, 0.78 Gm., plus magnesium carbonate, 0.07 Gm., plus magnesium trisilicate, 0.13 Gm.)

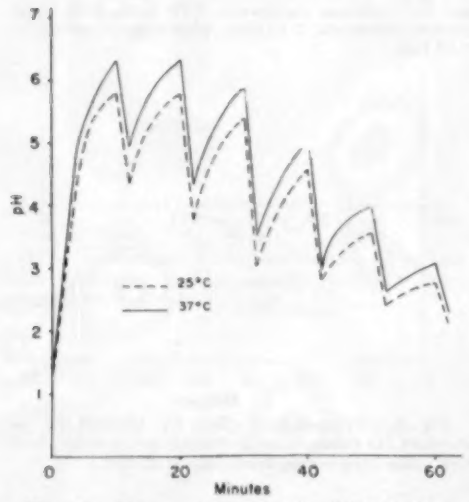

Fig. 10.—Temperature effect by Method IV on Product (*c*) (calcium carbonate, 0.78 Gm., plus magnesium carbonate, 0.13 Gm., plus magnesium oxide, 0.07 Gm.)

Method IV showed more difference on product (*c*) than did Method I. This may be the result of the emptying process in Method IV.

Reproducibility.—Typical results showing the ability to duplicate results on a single lot of product is shown in Fig. 15 and 16. Since product (*c*) generally has a higher *p*H range than products (*a*) and (*b*), these curves indicate a maximum of variability. The deviation from the mean in Method I at each point is relatively constant. In Method

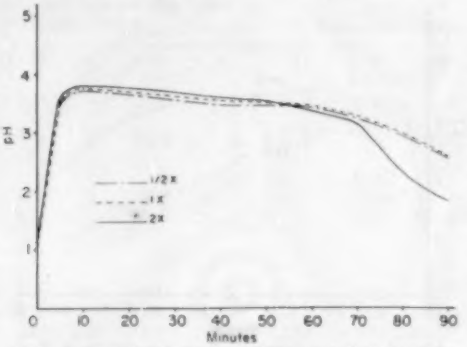

Fig. 11.—Stirring rate effect by Method I on Product (*a*) (aluminum hydroxide gel dried powder, 0.65 Gm., plus magnesium trisilicate, 0.32 Gm.)

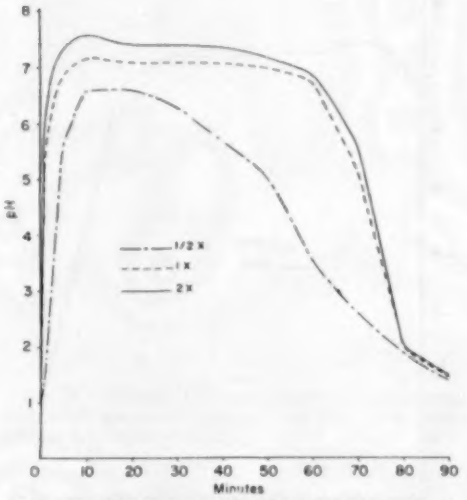

Fig. 12.—Stirring rate effect by Method I on Product (*b*) (calcium carbonate, 0.78 Gm., plus magnesium carbonate, 0.07 Gm., plus magnesium trisilicate, 0.13 Gm.)

IV, the peak values decrease in reliability as time progresses while minimum values increase. In each case, credence can be placed on the total curve only when several runs have been carried out.

Clemow and Lowry (11) have recently published a test for routine aluminum hydroxide evaluation. Our work on product (*a*) correlates closely with their report. Another method (12) employing continuous addition of hydrochloric acid and modifications of it are now being investigated. No comparative analysis of methods has previously been published.

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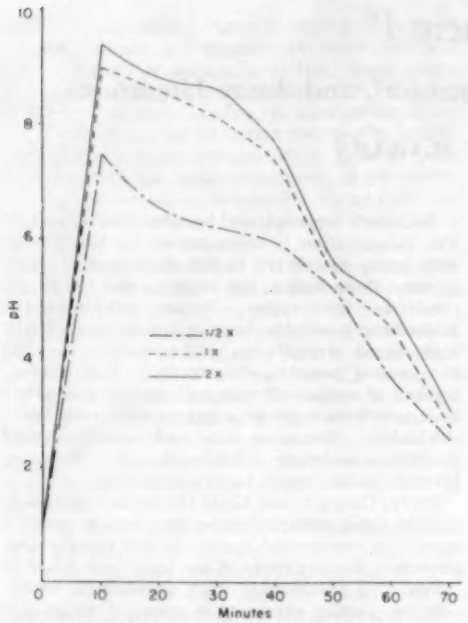

Fig. 13.—Stirring rate effect by Method I on Product (*c*) (calcium carbonate, 0.78 Gm., plus magnesium carbonate, 0.13 Gm., plus magnesium oxide, 0.07 Gm.)

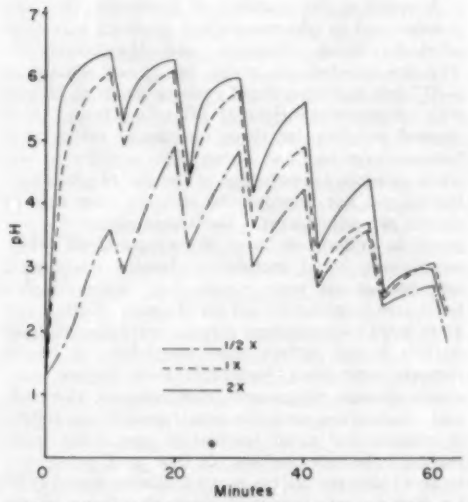

Fig. 14.—Stirring rate effect by Method IV on Product (*c*) (calcium carbonate, 0.78 Gm., plus magnesium carbonate, 0.13 Gm., plus magnesium oxide, 0.07 Gm.)

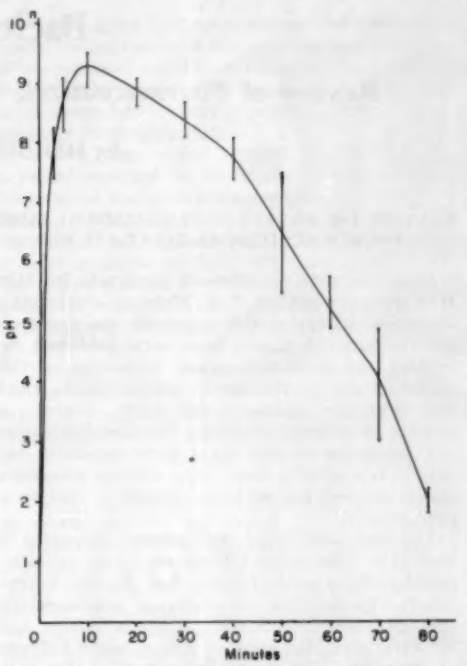

Fig. 15.—Reproducibility by Method I (5 runs) on Product (*c*) (calcium carbonate, 0.78 Gm., plus magnesium carbonate, 0.13 Gm., plus magnesium oxide, 0.07 Gm.)

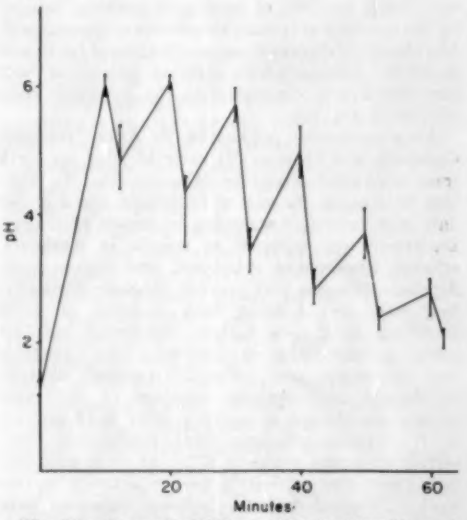

Fig. 16.—Reproducibility by Method IV (4 runs) on Product (*c*) (calcium carbonate, 0.78 Gm., plus magnesium carbonate, 0.13 Gm., plus magnesium oxide, 0.07 Gm.)

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Bacitracin I*

Review of Pharmaceutical, Chemical, and Assay Literature

By HERBERT M. GROSS

REVIEW OF BACITRACIN CHEMICAL AND PHARMACEUTICAL LITERATURE

Since the 1943 discovery of bacitracin by Miss B. A. Johnson and Dr. F. L. Meleney, considerable literature concerning this antibiotic has appeared. Several hundred papers have been published describing the chemotherapeutic properties of this medicinal and the therapeutic applications to which this efficacious antibiotic has been adapted. A number of articles concerning the chemical nature and properties of this agent have appeared, but only a few articles have been written describing pharmaceutical formulations, stabilities, and compatibilities.

The first publication on bacitracin appeared in 1945 (1). The article introduced a new antibiotic produced by a strain (Tracy I) of *Bacillus licheniformis*. In addition to reporting pharmacologic and chemotherapeutic data, Johnson, Anker, and Meleney began the series of papers which followed on the chemical and physical nature of this antibiotic. The active principle was described as neutral, water-soluble, and relatively heat stable. A fifteen-minute stability at 100° was reported. An article by Scudi, Coret, and Antopol (2) in 1947 mentioned the loss of bacitracin activity induced by the presence of sodium thiosulfate or dimercaprol. Fischbach (3) discussed the purification of bacitracin in 1948. Electrodialysis utilizing cellophane bags was effective in concentrating the antibiotic from 37.5 to 62.2 u./mg.

A comprehensive publication by Anker, Johnson, Goldberg, and Meleney (4) early in 1948 set forth some additional properties of bacitracin. In addition to defining the unit of bacitracin, considerable data were presented regarding bacitracin properties. Bacitracin was reported as soluble in methanol, ethanol, isopropanol, *n*-butanol, and cyclohexanol. Aqueous diffusion was possible through nitrocellulose membrane holding back material of 2,000 molecular weight or higher. Bacitracin was reported as adsorbable on charcoal, Lloyd's reagent, and aluminum oxide. Partially-purified, neutral, or slightly acid aqueous solutions of bacitracin showed no change in potency after 8–12 months at 5°. These solutions were inactivated completely after two weeks at 37°. At room temperature there was a 30–50% loss in activity in two weeks. Neutral inorganic salt-free solutions were dried at room temperature without activity loss. In a dry state in vacuum vials, bacitracin was stable at room temperature. Bacitracin solutions were stable to *N* HCl at 0 to 5° but not at 37° and to 0.01 *N* HCl at 3° and 37°. Alkaline solutions above *p*H 9 were rapidly inactivated at both temperatures.

Bacitracin is precipitated by salts of heavy metals. The precipitation is accompanied by inactivation with heavy metals low in the electromotive series, whereas those high in the series as zinc (5) do not result in inactivation. Tannic, trichloroacetic, azobenzene *p*-sulfonic, benzoic, furoic, and salicylic acids cause precipitation of bacitracin; the first two causing complete deactivation. High concentrations of sodium chloride and acetone precipitate bacitracin from aqueous solutions while ammonium rhodanilate, Reinecke salt, and molybdic acid precipitate bacitracin as a complex salt. Hydrogen peroxide causes loss in bacitracin activity.

Barry, Gregory, and Craig (6) studied bacitracin by the Craig countercurrent distribution method, separating commercial bacitracin into several components. Further study of the hydrolytic products indicated a polypeptide with approximate amino acid composition expressed in grams of amino acid per 100 grams bacitracin as phenylalanine 11, leucine 9, isoleucine 22, glutamic acid 10, aspartic acid 17, lysine 9, histidine 10, cystine 14, and ammonia 15. The following amino acids were reported as absent; methionine, valine, serine, threonine, proline, and arginine.

A study of the stability of bacitracin as a dry powder and in pharmaceutical products was compiled by Bond, Himelick, and Macdonald (7). The dry powder was stable for fifteen months at 5–37° but indicated rapid activity losses at 56 and 80°. Aqueous solutions at *p*H values from 5 to 7 showed stability for three months at refrigerated temperatures but lost about 50% activity in one week at room temperature. The use of phosphate buffers did not improve the stability over that of simple *p*H adjustment. Bacitracin ointments prepared in anhydrous bases of cottonseed oil, white petrolatum, liquid petrolatum, lanolin, white wax, and peanut oil were stable, but water-miscible bacitracin ointments prepared with Carbowax® 1500, 4000, (polyethylene glycol), cetyl alcohol, and sodium lauryl sulfate were nonstable. A troche formula containing bacitracin with lactose, saccharin sodium, tragacanth, cinnamon oil, clove oil, and magnesium stearate was reported as stable. A solution for nasal instillation containing bacitracin, ephedrine sulfate, lactose and phosphate buffers indicated full potency in solution stored at 5° for fifteen weeks and 85% retained activity stored at 25° for two weeks.

In 1949 a patent was issued to Goorley (8) assigned to Ben Venue Laboratories for the purification of bacitracin. Aqueous solutions of crude bacitracin were purified by forming a precipitate of inert impurities by the addition of ammonium sulfate. Additional ammonium sulfate added to the purified liquid precipitated bacitracin in purified water-soluble form.

Bacitracin was officially recognized under the Food, Drug, and Cosmetic Act, in July 1949, as a

* Received April 13, 1955, from The Pharmaceutical Research Division, Commercial Solvents Corp., Terre Haute, Ind.

certifiable antibiotic. Early in 1950 a patent was granted Johnson and Meleney (9) who dedicated their discovery of bacitracin to the United States of America. Newton and Abraham (10) reported the results of their studies on another antibiotic called ayfivin. Countercurrent distribution studies indicated the main component of ayfivin to be identical with the main component of bacitracin. Infrared studies on various antibiotics by Gore and Peterson (11) included the infrared spectrum of bacitracin.

Craig, Gregory, and Barry (12) performed purity studies on bacitracin and reported the active principle to be a polypeptide. Fractionation by countercurrent distribution techniques produced a purified product of 60 u./mg. and it was thought at that time that 66 u./mg. was the maximum potency of this medicinal. Hydrolysis and paper chromatography coupled with the work of fractionation resulted in isolation of amino acids; *l*-histidine, *dl*- and *l*-leucine, *l*-cysteine, *l*-glutamic acid, *dl*-phenylalanine, *dl*-aspartic acid, and *dl*- and *d*-isoleucine.

The chemical and physical properties of bacitracin were discussed in a biochemical engineering article in 1951 by Inskeep, Bennett, Dudley, and Shepard (13). They reported elementary chemical analyses revealed carbon, hydrogen, nitrogen, and sulfur occurring in bacitracin in comparable percentages as in a typical protein but revealed the absence of halogens or phosphorus. Bacitracin resisted hydrolysis by pepsin and trypsin and a variety of other proteolytic enzymes. It was readily hydrolyzed by acidic or basic solutions, particularly when heated. They stated that aqueous bacitracin solutions are most stable in the *p*H range 4 to 5.

Craig, Weisiger, Hausmann, and Harfenist (14) continuing their efforts to identify bacitracin, published information on their countercurrent distribution studies to indicate bacitracin to be a mixture comprising a main component, bacitracin A, and several minor components described as bacitracin B, C, D, E, and F. They isolated the major component and studied its ultraviolet absorption spectrum. This component was inactivated by alkali, strong acid, or formaldehyde. Subsequent investigation by Craig, Hausmann, and Weisiger (15) on the qualitative and quantitative amino acid content of bacitracin A indicated *d*-phenylalanine, *l*-leucine, *l*-isoleucine, *l*-cystine, *d*-glutamic acid, *dl*-aspartic acid, *l*-histidine, *l*-lysine and *d*-ornithine to be present and that aspartic acid and isoleucine were present in double molar ratios with respect to the other amino acids.

Newton, Abraham, Florey, Ross, and Smith (16) collaborated on an investigation of the biological properties of bacitracin A, B, and C. Crude bacitracin was fractionated by countercurrent distribution methods into relatively pure samples. Experimentation indicated mouse toxicities, on a weight basis, to be greatest with bacitracin C and least with bacitracin B. However, bacitracin B was less effective against various bacterial species than A and C. Data on bacitracin F were not included in this study. Later this fraction and its relation to bacitracin A in potency and toxicity proved to be important.

Porath (17) investigated bacitracin and reported some properties of purified bacitracin. Paper chromatography and electrophoresis methods confirmed former results on the amino acid constituents of bacitracin A and by an ultracentrifugal method the molecular weight of bacitracin A was calculated to be about 1,460. Porath proposed an amino acid sequence for bacitracin A.

In 1951 Hegarty and Verway (18) were granted a patent assigned to Sharp and Dohme claiming bactericidal therapeutic compositions of ointments, lotions, and tablets containing bacitracin with tyrothricin or streptomycin. Charney (19) was granted a patent in 1952 which was assigned to Sharp and Dohme for a purification method for bacitracin which involved adsorption of bacitracin from solution on zeolitic materials. A patent was granted Welch (20) for therapeutic compositions of liquids, tablets, capsules, and suspensions containing bacitracin with polymyxin and streptomycin. A patent pertaining to the therapeutic composition of products containing bacitracin and neomycin was granted Felsenfeld (21) and assigned to Commercial Solvents Corp.

A study of the effects of surface-active agents on bacitracin activity by Bruce and Mitchell (22) indicated that bacitracin was enhanced by the presence of cationic and nonionic surface-active agents and retarded by anionic agents. The combination of bacitracin with quaternary ammonium compounds was suggested for therapeutics in which tissue penetration of the antibiotic is desirable.

Jeffries, *et al.* (23), published an article in 1952 on an antibiotic ointment base suitable for bacitracin. The antibiotic was reported as stable in this water-diffusible ointment base consisting of polyethylene glycol 400 distearate, polyethylene glycol 400 dilaurate, glyceryl tristearate, and petrolatum.

Several articles were published in 1953 concerning the structure of bacitracin. Newton and Abraham (24) investigated the structure of bacitracin A reporting it as a polypeptide containing the amino acids; cysteine, ornithine, lysine, histidine, aspartic acid, glutamic acid, phenylalanine, leucine, and isoleucine. They reported a molecular weight of about 1500. Results of their electrometric titrations and reactions indicate that bacitracin A contains two free carboxylic acid groups, one free α-amino group, one histidine glyoxaline structure, and one free δ-amino group of ornithine. The ϵ-amino group of lysine is not free. It was suggested that bacitracin A has a cyclic formation in which the carboxylic acid group is condensed with the ϵ-amino group of lysine. They proposed a possible thiazoline ring system. A second paper (25) by the same investigators indicated that by use of countercurrent distribution techniques they had resolved crude bacitracin into at least 10 polypeptides; E, D, B, A′, A, C, G, F_1, F_2, and F_3. They performed ultraviolet spectrum determinations on the fractions and indicated maximums at various wavelengths. They concluded that all bacitracins contained: cysteine, ornithine, lysine, histidine, aspartic acid, glutamic acid, leucine, and/or isoleucine, and phenylalanine. Bacitracins B, D, and E yield a substance similar to valine, and bacitracin C yielded a substance similar to glycine. They confirmed the statement that no free thiol groups existed but that they were liberated from all bacitracins upon hydrolysis. Ammonia was liberated

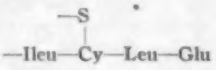
from amide groups in bacitracins B, A, C, G, F_1, F_2, and F_3.

As a result of studies by method of partial substitution, Craig, Hausmann, and Weisiger (26) reported the molecular weight of bacitracin to be about 1,470. They further added that bacitracin A contained two free amino groups, one being an ornithine. Bacitracin A reportedly was transformed at *p*H 7 or higher to an F-type bacitracin of extremely low potency.

In an investigation by Ingram (27) concerned with the identification of peptide end groups, bacitracin was methylated and hydrolyzed and analyses performed to conclude that no free α-amino groups existed in the molecule to confirm the observation that one δ-amino group of ornithine was free.

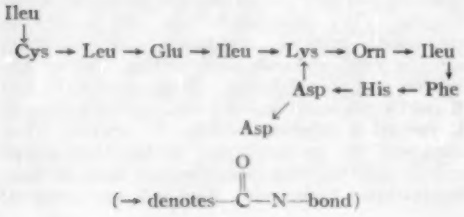
A stability study of aqueous solutions of bacitracin (28) appearing in 1954 indicated that prolonged exposure of bacitracin solutions to light resulted in pronounced inactivation. Solutions of bacitracin stored at 20° protected from light and air movements remained active. Similar results were effected in atmospheres saturated with nitrogen. Oxygen had only a secondary influence on inactivation as compared with the reaction due to light.

Further studies concerning the structure of bacitracin A by Lockhart, Newton, and Abraham (29) yielded partial hydrolysis products as dipeptides, tripeptides and tetrapeptides which indicated the following structural amino acid sequence to be present in bacitracin A:

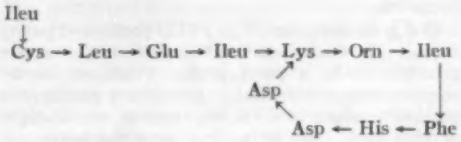


Additional results suggested the left side of the molecule could be extended through a peptide containing ornithine, phenylalanine, and isoleucine with ornithine as terminal amino acid and that the right side of the sequence contained glutamic and aspartic acids closely associated. The molecule appeared to be a cyclic chain with the polar side chains on the other moiety. Chromatographic and ionophoretic studies by Lockhart and Abraham (30) suggested a tentative unknown linkage between phenylalanine and the isoleucine residue adjacent to cysteine. Peptides from commercial bacitracin which indicate valine to be present suggest that the amino acid sequence lys.-orn.-val.-phe. occurs in bacitracin B. Further observations on the structure of bacitracin A by Lockhart and Abraham (31) indicate the probability that the isoleucine adjacent to cysteine is *allo*isoleucine.

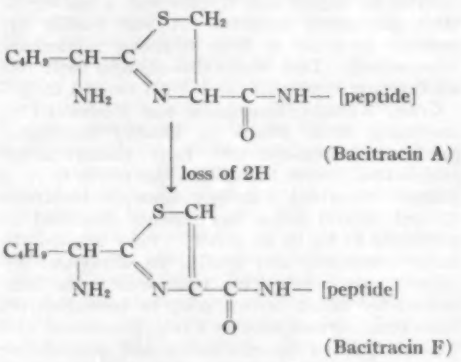
Continued structural studies on bacitracin A by Craig, Hausmann, and Weisiger (32, 33) indicated bacitracin A to contain 3 isoleucine residues rather than the two formerly theorized. An overall formula $C_{66}H_{103}O_{16}N_{17}$ has been proposed as well as the amino acid sequence:



These results concur with Lockhart, et al. (29), but not with Porath (17). A study of partial hydrolysis products of bacitracin A by countercurrent distribution, paper chromatography, and zone electrophoresis has yielded information on a large number of peptides and derivatives which have been isolated and characterized. In a later article (34), the authors indicate a formula which also must be considered as:



An investigation by Codington (35) on the relationship between the biological properties and composition of bacitracin revealed some interesting information which correlates with previously reported literature. Special significance was made to the presence of the thiazoline ring in the molecule as previously reported by Newton and Abraham (24). Codington felt that this structure was closely related to the therapeutic activity of bacitracin. Chemical and physical evidence indicated the conversion of bacitracin A to F may involve the transformation of a thiazoline ring to a thiazole ring as indicated:



On the basis of biological studies Codington concluded that acute lethal toxicities may be attributed to the relative amounts of bacitracins A and F. On a weight basis bacitracin F is of the same order of toxicity as bacitracin A; but because of the low antibiotic activity of bacitracin F, on a unit basis bacitracin F is more toxic. He reported activity of bacitracin A as high as 67.3 units per milligram and bacitracin F as low as 2.7 units per milligram. Bacitracin F was reported very stable. Bacitracin A in dry form at room temperature was very slowly converted to bacitracin F, approximately 15–20% in two years. However, in solution the change was much accelerated and specifically influenced by alkalinity. At *p*H 7.0 at 25° the transformation was complete in twenty-two days, but only a 5% transformation was noted after twenty-four days at 0°.

Weisiger and co-workers (36) studied the partial hydrolysis products of the dinitrophenyl derivative

of bacitracin A by isolation and characterization of the peptides produced. The data presented correlate with their previously proposed amino acid sequence for bacitracin A.

Welch (37) described the various bacitracin dosage forms which are acceptable to the F. D. A. and discussed the pharmacology and chemotherapy of this antibiotic. Another book (38) including the ingredients, trade names, indications, and manufacturers of bacitracin products has been published. Baker (39) listed published data on pharmaceutical formulations of bacitracin and presented information relative to the therapeutic applications of bacitracin and its synergistic combinations as reported by clinicians. A recent publication (40) discussed the formulation, compatibility, and stability of bacitracin products.

REVIEW OF BACITRACIN ASSAY

Anker, Johnson, Goldberg, and Meleney in their report (4) on the new antibiotic bacitracin, defined the unit of bacitracin as the amount which, when diluted 1:1024 in a series of twofold dilutions in 2 cc. beef infusion broth completely inhibits the growth of a stock strain of group A hemolytic streptococcus when inoculum used to seed the tubes is 0.1 cc. of a 10^{-2} dilution of an overnight culture in blood broth. They described a serial dilution assay.

Hoff, Bennett, and Stanley (41) described a cylinder plate assay which allowed routine handling techniques. The method utilized the organism *Micrococcus flavus* (ATCC No. 10240) to produce sharper zones of inhibition with smooth growth characteristics. Some variables in this method of assay were outlined by Pinzelik, Nisonger, and Murray (42). In use of the agar diffusion bioassay, delay in addition of sample to prepared assay plates reportedly resulted in smaller zones of inhibition, especially if plates were refrigerated. It was therefore essential that standards and samples to be assayed be added at the same time to the plates. Adaptations of this method for microbiological assay of bacitracin and neomycin combined in galenical pharmaceuticals were described by Trolle-Lassen (43).

A study correlating zones of inhibition on agar plates with minimal inhibitory concentrations of bacitracin as determined by the serial dilution method was made by Patrick, Craig, and Bachman (44). The comparison was made by using antibiotic-containing tablets on 58 bacterial strains. Howe (45) evaluated the paper disk method of analysis with results obtained by serial tube dilution methods; greater antibiotic activity was indicated in serial dilution methods. Bond and Nook (46) described a plate assay method adapted to the assay of bacitracin in body fluids which would detect bacitracin concentration as low as 0.02 u./ml. of fluid. Darker, *et al.* (47), compared two methods of bacitracin analysis discussing the cylinder plate assay and the turbidimetric method as well as discussing the numerical calculations relative to antibiotic activity and zones of inhibition. Landgren (48) described the identification of various antibiotics including bacitracin by determination of their refractive indices. Hadfield (49) prepared frozen suspensions of *M. flavus* for use in the microbiological assay of bacitracin and reported on determinations using these preparations.

"Compilation of Regulations for Tests and Methods of Assay and Certification of Antibiotic and Antibiotic-Containing Drugs," Volumes I and II, section 141e.401 and following paragraphs describe the official methods of assay accepted by the Food and Drug Administration.

An International Standard for bacitracin was defined by Humphrey, Lightbown, Mussett, and Perry in the *Bulletin of the World Health Organization* (50). The standard accepted has an antibiotic activity of 55 u./mg. of bacitracin.

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Bacitracin II*

Stability Characteristics

By HERBERT M. GROSS

The stability of bacitracin at various temperatures is reported. A comparison of the stabilities of high and low potency lots of bacitracin indicated no significant difference existed.

REALIZING THE NEED for a general upgrading of the antibiotic and responding to the urgings of clinical investigators such as Meleney (1) for a purified product, the industry has developed and adopted many process innovations to yield a more highly refined bacitracin. The desire of the clinician to use as high a potency material as possible is logical and substantiated by toxicity data (2, 3). A convenient yardstick to measure these technologic improvements is potency per unit weight of antibiotic. Over a period of five years, the number of units of bacitracin activity per milligram of drug has risen from 30 to over 75. Bacitracin potency has been a frequent topic of publication by clinical as well as chemical investigators. Parenteral bacitracin, established as a certified drug product by the Food and Drug Administration, was required in January 1952, to possess a minimum potency of 50 u./mg. activity. Pharmaceuticals designed for topical or oral administration were allowed to be prepared with bacitracin of 30 u./mg. activity, and the antibiotic was defined as a 40 u./mg. product. This set the triple standard for bacitracin which has been adopted in U. S. P. XV. However, in light of current manufacturing and sales practices, this triple standard appears to be unnecessary. Bacitracin is sold on a 50 u./mg. basis and only rarely do purchasers of bulk antibiotics accept lots of lesser potency. Bacitracin literature reveals several statements estimating the ultimate potency of pure bacitracin to be 60 or 66 u./mg. (4, 5). These supposed maximum potencies have been exceeded and stable bacitracin of higher potency has been prepared commercially.

With the advent of a higher potency bacitracin, came the predictable drop in impurities such as ash, insoluble organic material, heavy metals, and water. Toxicities diminished because less material was necessary to exert the required antibiotic activity (3). The physical properties also improved in that clarity of solution, ready solubility, and product uniformity were possible. The aqueous solubility of bacitracin A, the active bacitracin molecule, is many times greater than bacitracin F which is an inactive degradation product of bacitracin A (2). In 1953 the Food and Drug Administration released bacitracin for use by physicians outside of the hospital as well as in the hospital, considering it to be safe for intramuscular or intravenous use. The Council on Pharmacy and Chemistry of the American Medical Association recognized the use of parenteral bacitracin in 1954 and it appeared in a monograph of "New and Nonofficial Remedies."

Naturally, as process improvements and purification procedures were developed and introduced, the possibility of a variance in therapeutic activity and chemical stability had to be considered. To check these possibilities, testing followed processing advances. Characterization tests which studied the therapeutic uniformity of bacitracin by determining the activity of the antibiotic against specific microorganisms will be discussed in another publication. This article deals with the studies which tested the uniformity of bacitracin potency stability. The stability of the antibiotic is directly related to the stability, compatibility, and formulation of bacitracin products (6).

EXPERIMENTAL

Stability Characteristics of Bacitracin.—The initial potency and moisture content of lots of bacitra-

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cin representing production from 1951 through 1954 were obtained. The true activities of these lots were calculated by correction of the assayed potencies for moisture. The moisture content of lots varied between 1.8% and 5.0%, averaging 3.5%. Samples of these lots were stored in various tests for two-year periods with interim determinations at one-, two-, three-, six-, twelve-, and eighteen-month intervals. Storage temperatures of 5, 10, 25, 40, and 60° were used. Potency determinations were accompanied by moisture assays and actual activities obtained by correction. The results of these tests are graphically demonstrated in Fig. 1 by thermal stability characteristic curves derived from the mean retained potencies of various lots at assay intervals. All bacitracin activities were determined by the microbiological plate-assay method (7). Moisture determinations were made by a gravimetric method. Samples were weighed, dried under vaccum at 60° for three hours, and reweighed. This loss on drying was termed moisture content. The Karl Fischer method cannot be used for bacitracin moisture determinations since bacitracin reacts with the reagent to form iodobacitracin.

Stability of High and Low Potency Lots of Bacitracin.—Over a three-month period, the retained potencies of 29 random bacitracin lots varying in potency from 45 to 59 u./mg. were compared with 9 random lots of 63 to 71 u./mg. activity. Antibiotic potency and moisture content were determined at various intervals as described above. The true potencies were calculated and mean retained activities for high and low potency lots were plotted in Fig. 2. A curve describing the stability characteristic of bacitracin was evolved from these data.

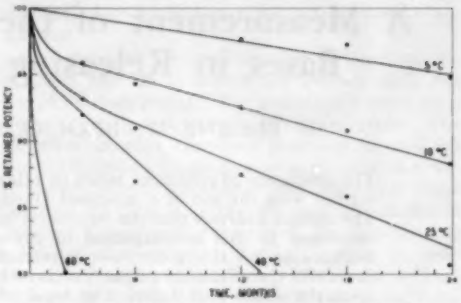

Fig. 1.—Stability characteristics of bacitracin.

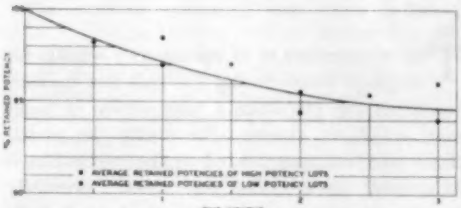
% RETAINED POTENCY
AVERAGE RETAINED POTENCIES OF HIGH POTENCY LOTS
AVERAGE RETAINED POTENCIES OF LOW POTENCY LOTS
TIME, MONTHS

Fig. 2.—Stability characteristic of high and low potency lots of bacitracin.

DISCUSSION

The thermal stability characteristics of bacitracin as graphically demonstrated in Fig. 1 call to attention the inactivating effects of time and temperature on bacitracin. A characteristic minor, initial drop in potency was exhibited within the two-month interval. Although this initial decrease in potency is slight, it is an established characteristic of bacitracin stability. However, the degree of inactivation indicated is not of the magnitude previously accepted. After this slight initial drop in activity, the stability follows a definite trend which is a function of time and temperature. The concept that the potency of bacitracin will drop, for example, from 55 to 40 u./mg., a 27% loss of activity, in a brief time and then remain stable at that level has been negated by the data presented. The activity loss at 25° would approach only 6% in two months.

By pin-pointing the stability characteristics of high potency bacitracin and low potency bacitracin over a three-month period, Fig. 2 has revealed that the percentage retained activities of the two potency level products are the same. The deviation of mean retained potencies from the stability characteristic curve, which actually represents the stabilities of the lots at 10°, is never more than 1.5%. It has been stated that as the purity (potency) of bacitracin is increased toward its theoretical potency, its stability tends to decrease (4). This study of high and low potency bacitracin lots refutes this theory. The stability of high potency bacitracin does not differ significantly from the stability of low potency bacitracin.

These slight variations in stability characteristics were considered to be within the limits of the tests employed. Several factors influence the accuracy of stability data obtained for bacitracin and should be considered in its evaluation. The activity determination for bacitracin is a cylinder-plate assay subject to the variables of a microbial method. Assay results may vary by ±5%. To reduce such variations to a minimum, triplicate three-plate determinations on three successive days were used.

SUMMARY

The stability characteristics of bacitracin at 5, 10, 25, 40, and 60° have been demonstrated.

A comparative study between lots of high potency bacitracin (63–71 u./mg.) and lower potency bacitracin (45–59 u./mg.) indicated no significant difference in stabilities.

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A Measurement of the Efficiency of Ointment Bases in Releasing a Local Anesthetic*

By EUGENE W. BROCKEMEYER† and EARL P. GUTH

The efficiency of ointment bases in releasing a local anesthetic was studied on the rat tail with the aid of a modified Hardy-Wolff-Goodell pain threshold apparatus. The results indicate that the bentonite bases are more efficient than the other bases employed in this investigation in providing not only a rapid release of cyclomethycaine but also a continuous release of this anesthetic for four hours. Aging decreases the efficiency of the Volclay® bentonite ointment whereas it has no effect upon the depth and duration of local anesthesia produced by hydrogen bentonite ointment.

THE INTRODUCTION of numerous formulas for ointment bases during the past few decades has given the physician a wide choice of bases from which to choose. Previously the physician's selection was limited to the grease bases. As a result, his attention was centered primarily on the medicament and its effect, and little thought was given to the selection of a proper base for the medicament.

Most of the methods reported in the literature for evaluating the efficiency of ointment bases are designed to measure the absorption of bactericidal and fungicidal agents, antibiotics, hormones, vitamins, dyes, alkaloids and various poisons, inorganic salts, and radioactive tracer compounds. The work of Christensen and Tye (1) in 1951 on analgetics and later the work of Mack, Nelson, and Guth (2) and Lucas and Guth (3) in 1953 suggested a pain threshold method utilizing a modified Hardy-Wolff-Goodell pain threshold apparatus for the evaluation of ointment bases containing local anesthetics.

This study was made to ascertain the possible efficiency of a modification of the method suggested by the above investigators by determination of the depth and duration of anesthesia of the rat tail, produced by a local anesthetic incorporated in ointment bases.

EXPERIMENTAL

Apparatus Used.—The Hardy-Wolff-Goodell pain threshold apparatus as modified by Tye (4) in his work with analgetics was employed.

Local Anesthetic Used.—Cyclomethycaine sulfate[1] (Surfacaine®) was the local anesthetic used throughout this study. It belongs to a group of substituted piperidinoalkyl benzoates and is a white, crystalline, odorless powder, soluble in water to the extent of slightly more than 1 per cent. Cyclomethycaine is not affected by exposure to air nor sunlight and has a melting point of 162.5–165.5°.

Ointment Bases Used.—White ointment U. S. P. XIV, a grease type base; Surfacaine® ointment,[1] a w/o emulsion type base; hydrophilic ointment U. S. P. XIV, an o/w emulsion type base; polyethylene glycol ointment U. S. P. XIV, a water-soluble type base; and Volclay® bentonite base and hydrogen bentonite base, water-washable and fat-free bases, were selected for this study in order to have a representative from each different type of ointment bases. Two different bentonite bases were chosen to represent the water-washable and fat-free type of ointment base in order to supplement the information already obtained by the laboratories of the College of Pharmacy at The Ohio State University on the efficiency of ointment bases containing bentonite as a major component.

The Volclay® bentonite base was prepared according to the following formula which was proposed by Darlington and Guth (5):

	% w/w
Volclay bentonite[2]	20.0
Glycerin	10.0
Distilled water	70.0

The hydrogen bentonite base was prepared according to the formula proposed by Barr and Guth (6). However, the base seemed somewhat stiff after the local anesthetic was incorporated; so the formula was modified as follows:

	% w/w
Hydrogen bentonite	35.0
Glycerin	10.0
Distilled water	55.0

The hydrogen bentonite was prepared according to the method used by Barr and Guth (7).

To prevent the growth of molds, 0.15% methylparaben U. S. P. XIV and 0.05% propylparaben U. S. P. XIV were added to each of the bentonite bases.

It was observed that during the preparation of the bentonite bases, a considerable amount of water was lost due to evaporation. After the bentonite

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[1] The authors are indebted to Eli Lilly and Company for supplying the Surfacaine and Surfacaine Ointment.

[2] American Colloid Co., Chicago, Ill.

bases were prepared, they were weighed and the amount of water which had been lost was incorporated into the bases.

Experimental Animals.—Conditioned male albino rats (C. F. Wistar) weighing 100 to 200 Gm. were used. The feeding of the animals and the humidity and temperature of the animal room were kept as uniform as possible throughout the investigation. The normal pain threshold for the 50 rats used varied from 195 to 315 mc./sec./cm.^{2} The average normal pain threshold of the rats was 252 mc./sec./cm.^{2} and the average deviation from the mean normal pain threshold was 19 mc./sec./cm.^{2}

Preliminary Tests.—These tests showed that ointments containing 1% cyclomethycaine produced only a light degree of local anesthesia whereas ointments containing 5% cyclomethycaine produced a marked degree of local anesthesia. Therefore, cyclomethycaine in a concentration of 5% (w/w) was used throughout this study.

Also, it was shown by preliminary tests that the quantity of ointment applied to the tail was immaterial as long as an excess was applied. Four minutes was a satisfactory time period for applying the ointment on the tail. A moderate friction was used.

Determination of the Depth and Length of Local Anesthesia Produced by Cyclomethycaine in Various Ointment Bases.—An excess amount of ointment containing Surfacaine® was applied to the posterior one-third of the tail, which had previously been washed. After one-half hour, the ointment was removed as gently as possible and the test area painted with India ink. When the ink had dried, the pain threshold of each rat was determined. The pain threshold was redetermined at intervals of 1, 2, 4, 6, and 8 hours after the removal of the ointment. The elevation in pain threshold from the normal was used as the determinant for measuring the depth of local anesthesia. It was also possible to note the duration of local anesthesia by comparing the elevation in pain threshold at the various time intervals. Six animals were used to measure the efficiency of each ointment. Pain-threshold increase was also determined using the ointment bases without the anesthetic as a control.

The results showed that a high degree of local anesthesia was produced by the ointments prepared from bases containing water and that the depth of anesthesia produced by these ointments had decreased considerably during one hour. After eight hours, local anesthesia was slight. The ointment prepared with polyethylene glycol ointment U. S. P. XIV as the base had produced only a slight degree of local anesthesia when it was removed. However, the depth of local anesthesia in the animals continued to rise for four hours and then it gradually decreased during the next four hours. Very little, if any, local anesthesia was produced by the ointment prepared with white ointment U. S. P. XIV as the base.

Since in actual therapy a local anesthetic ointment is not usually removed but allowed to remain on the skin for a long period of time, it appeared that the procedure used did not give a true evaluation of the depth and duration of local anesthesia that these ointments were capable of producing. To obtain an evaluation of these ointments under conditions which more closely simulate clinical use, the following modification to the procedure was adopted.

The ointment was applied to six different groups of three animals each and the contact time was varied for each group. The periods of contact used were 1/4, 1/2, 1, 2, 3, and 4 hours respectively. The elevation in pain threshold produced in each rat was determined immediately after the ointment was removed. Each experiment was repeated three times on the same rat with three to four days of rest between experiments to prevent the possibility of tolerance development. The elevations in pain threshold found on the three different days were added together for each rat and the mean value was considered to be the elevation in pain threshold for that rat.

The ointment prepared with white ointment U. S. P. XIV as the base was not included in this experiment since it failed to produce any noticeable degree of local anesthesia in the previous experiment. Since Surfacaine® ointment contains only 1% w/w Surfacaine,® an additional 4% w/w of Surfacaine® was incorporated into the ointment in order that all of the ointments used in this experiment should contain the same concentration of the local anesthetic.

Figure 1 shows the comparison between the average rise in pain threshold for the three animals after the various contact periods produced by 5% w/w cyclomethycaine in the five different ointment bases used in this experiment. Cyclomethycaine produced its greatest depth of local anesthesia when incorporated into the bentonite bases. Hydrogen bentonite base was more efficient in releasing Surfacaine® than Volclay® bentonite base. The high degree of local anesthesia produced by the local anesthetic in the bentonite bases was still present after the ointments had been in contact with the test area for four hours. The depth of local anesthesia produced by the ointment prepared from hydrophilic ointment decreased rapidly after the ointment had been in contact with the tail for one-half hour. Ointments prepared from polyethylene glycol ointment U. S. P. XIV and Surfacaine® ointment as the bases produced their highest degree of local anesthesia after being in contact with the test area for three hours.

Since it is widely accepted that bentonite is capable of undergoing cationic exchange with other cations, the question arose would these bentonite ointments still produce the same degree of local anesthesia after standing for five to ten days.

With the procedure used in the previous experiment it was found that 5-, 8-, and 10-day old Volclay® bentonite ointment and hydrogen bentonite ointment raised the pain threshold to about the same degree as fresh preparations. Therefore, the values obtained with both the fresh and the old ointment were added up for each rat, and the mean value was considered to be the elevation in pain threshold produced in the rat by the 5–10 day old bentonite ointment.

The results showed that Volclay® bentonite ointment after standing for 5–10 days produces a lower degree of local anesthesia than when the ointment is freshly prepared. Aging of hydrogen bentonite ointment up to ten days had no effect upon the depth and duration of local anesthesia produced by this ointment.

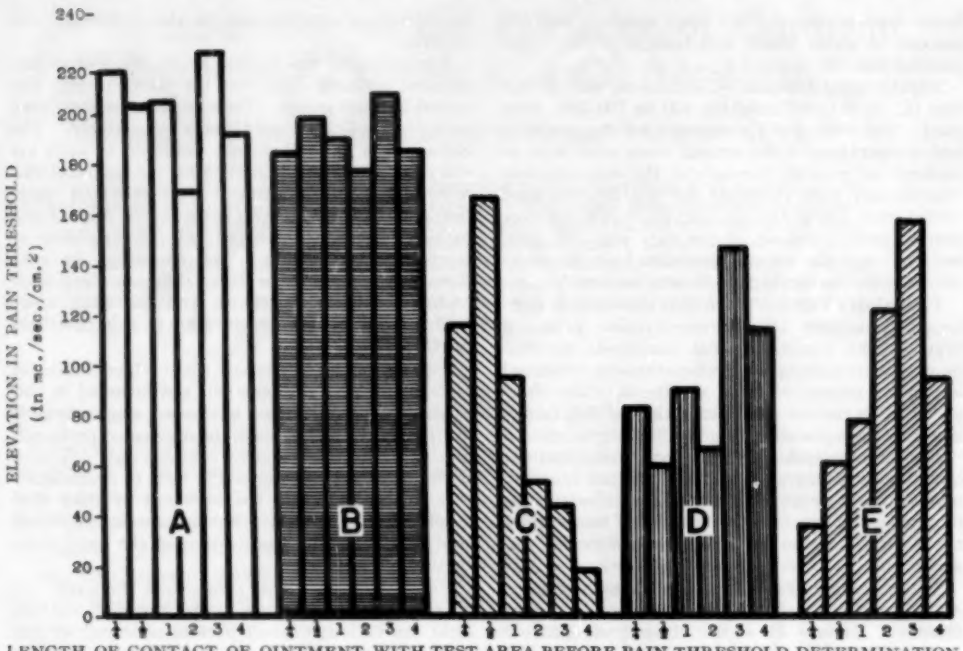

Fig. 1.—Comparison of the depth and duration of local anesthesia produced by 5% w/w cyclomethycaine in various ointment bases. Key: (A) Hydrogen bentonite base, (B) Volclay® bentonite base, (C) hydrophylic ointment, (D) polyethylene glycol ointment, and (E) Surfacaine® ointment.

The effect of age on the *p*H of the bentonite bases and the ointments prepared from them was determined since aging of the Volclay® bentonite ointment affected the depth of local anesthesia produced. The results are recorded in Table I.

TABLE I.—THE EFFECT OF AGE ON THE *p*H OF THE BENTONITE BASES AND OINTMENTS

Base or Ointment	Age in Days	*p*H
Volclay bentonite base	0	8.20
Volclay bentonite base	1	8.20
Volclay bentonite base	5	8.20
Volclay bentonite ointment	0	2.65
Volclay bentonite ointment	1	2.70
Volclay bentonite ointment	5	3.00
Hydrogen bentonite base	0	4.00
Hydrogen bentonite base	1	4.00
Hydrogen bentonite base	5	4.00
Hydrogen bentonite ointment	0	1.65
Hydrogen bentonite ointment	1	2.00
Hydrogen bentonite ointment	5	2.20

The addition of cyclomethycaine to the bentonite bases resulted in a significant change in *p*H. The *p*H of the Volclay® bentonite base dropped from 8.20 to 2.65 upon the incorporation of 5% cyclomethycaine, while the *p*H of the hydrogen bentonite base dropped from 4.00 to 1.65.

The *p*H of the hydrogen bentonite ointment after standing for five days had increased 0.55 of a *p*H unit, whereas the *p*H of the Volclay® bentonite ointment during the same time had increased only 0.35 of a *p*H unit. The *p*H of the 5-day old hydrogen bentonite ointment was lower than the *p*H of the Volclay® bentonite ointment when freshly prepared. A 1% aqueous solution of the local anesthetic gave a *p*H of 1.65, the same *p*H as that of the hydrogen bentonite ointment when freshly prepared.

DISCUSSION

The results obtained substantiate the belief that ointment bases do not possess the same degree of efficiency in releasing an incorporated medicament. The ability of the six bases used in this study to release cyclomethycaine varied widely.

The ointments prepared from bases containing water produced a greater degree of local anesthesia than the ointments prepared from bases containing no water. It is believed that the rate of release of a medicament from a base is greatly influenced by the solubility of the medicament in the external phase of the base. However, it can be concluded from Fig. 1 that the percentage of water present is not the sole determinant in the rate of release of cyclomethycaine since hydrogen bentonite base containing 55% water, was more efficient than Volclay® bentonite base which contains 70% water.

The rate of release of the local anesthetic from polyethylene glycol ointment closely paralleled the rate of release of the local anesthetic from the commercial ointment. No water was used in preparing the ointment with polyethylene glycol and it is presumed that no water is present in the commercial ointment. Therefore, it appears that the presence of water in a base increases its efficiency in releasing the local anesthetic.

The depth and duration of local anesthesia produced by the drug when incorporated in bentonite ointments was remarkable. A greater depth of anesthesia might have been produced by these ointments than indicated in Fig. 1 since it was impossible to obtain a positive response with many of the rats when the maximum stimulus possible with the Hardy-Wolff-Goodell apparatus was applied. The maximum stimulus that could be applied with this apparatus was 450 mc./sec./cm.^{2} When one takes into account that previous investigators (1–3) considered 325–250 mc./sec./cm.^{2} to represent a highly satisfactory degree of local anesthesia, the results obtained with the bentonite ointments are even more outstanding. If, after the maximum stimulus had been applied four times, the rats did not respond, 460 mc./sec./cm.^{2} had to be regarded the pain threshold. Since 460 mc./sec./cm.^{2} had to be considered as the elevated pain threshold for most of the rats after the removal of the bentonite ointments, the depth of anesthesia produced by these ointments as shown in Fig. 1 appeared a little uneven. This unevenness in the depth of anesthesia was due to the fact that the normal pain threshold of the rats varied slightly among the different groups used.

In order to get a true measurement of the depth of local anesthesia produced by the bentonite ointments, one would need to decrease the concentration of the local anesthetic in these ointments. However, the object of this research was to make a direct comparison between the efficiency of the various ointment bases and a 5% concentration of the local anesthetic was necessary in the less efficient bases in order to obtain a measurement of the release of the anesthetic.

An attempt was made to obtain information as to the difference in the release of the cyclomethycaine from Volclay® bentonite ointment when freshly prepared and after standing for 5–10 days. Perhaps the cyclomethycaine had undergone cationic exchange with the bentonite in the Volclay® bentonite ointment on standing since it is known that the cations of bentonite may be replaced by organic cations as well as inorganic cations. Although hydrogen bentonite ointment produced the same depth and duration of local anesthesia after standing for 5–10 days as it did when freshly prepared, it is still possible that some degree of cationic exchange took place between the cyclomethycaine ion and the hydrogen ion. Therefore, perhaps it is the degree of cationic exchange that accounts for the difference in the rate and amount of release of the local anesthetic from the two bentonite ointments after standing.

If the cyclomethycaine did undergo cationic exchange with the Volclay® bentonite base, it appears from the results shown in Table I that the exchange took place slowly since the *p*H of the ointment had changed only 0.05 of a *p*H unit after 1 day and 0.35 of a *p*H unit after 5 days of aging.

The observation that hydrogen bentonite base is superior to Volclay® bentonite base in releasing cyclomethycaine parallels the findings of Barr and Guth (6) in antiseptics and those of Lucas and Guth (3) in local anesthetics.

No explanation can be given for the decrease in local anesthesia produced by 5% w/w cyclomethycaine in polyethylene glycol ointment U. S. P. XIV after it had been in contact with the rat's tail for one-half and two hours as shown in Fig. 1.

CONCLUSIONS

1. The bentonite bases are more efficient in releasing cyclomethycaine than hydrophilic ointment, polyethylene glycol ointment, white ointment, or Surfacaine® ointment.

2. The bentonite bases give a rapid and continuous release of cyclomethycaine for four hours.

3. The hydrogen bentonite base is more efficient in releasing cyclomethycaine than Volclay® bentonite base.

4. Aging the Volclay® bentonite ointment decreases the depth of local anesthesia produced by this ointment; whereas aging of the hydrogen bentonite ointment has no effect on the depth or duration of local anesthesia.

5. The addition of cyclomethycaine to the bentonite bases produces a significant lowering in the *p*H of the bases.

6. Cyclomethycaine is more rapidly released from ointment bases that contain water than from those without water.

7. The percentage of water present in the ointment bases is not the sole determinant in regulating the rate of release of the local anesthetic.

8. The rate of release of cyclomethycaine from polyethylene glycol ointment more nearly approximates the rate of release of cyclomethycaine from grease type ointment bases than from bases containing water.

9. White ointment is inefficient in releasing cyclomethycaine.

10. The efficiency of ointment bases in releasing a local anesthetic can be determined by using a modification of the Hardy-Wolff-Goodell pain threshold apparatus.

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A Note on the Alkaloidal Ratios in Certain Species of Datura*

By RIAD R. ALAMI†, BERNARD V. CHRISTENSEN, and JACK L. BEAL

A comparative study was made of *D. stramonium* L., *D. stramonium* L. var. *inermis*, *D. tatula* L. It consisted of the determination of the total and individual alkaloidal contents of the leaves. Paper chromatography was the method employed in separating the total alkaloids into the individual components which were subsequently assayed. Known sample mixtures of pure hyoscyamine and scopolamine were separated by the use of paper electrophoresis at a *p*H of 9.5 after a two-hour period of electromigration.

OF THE MANY different genera of the Solanaceae family probably those species belonging to the genus *Datura* have been the subject of the most contradictory writings. Much of the incongruity has been in the alkaloid data of the plants. The two official sources of Stramonium, *Datura stramonium* L. and *Datura tatula* L., are good examples of this variance in alkaloid data. For example, Rowson has reported (1) the scopolamine-hyoscyamine ratio (S-H ratio) for *D. stramonium* to be 1:7.40 and for *D. tatula* to be 1:4.90. On the other hand, Evans and Partridge reported (2) the S:H ratio for *D. stramonium* to be 1:3.22 and for *D. tatula* to be 1:0.62. Probably a great amount of this variance is due to differences in environmental factors such as weather and soil.

This paper is an abstract reporting data of the alkaloidal content of *D. stramonium* L., *D. stramonium* L. var. *inermis*, and *D. tatula* L. grown in the Medicinal Plant Garden of the College of Pharmacy, The Ohio State University. In addition, a report is given on some preliminary work using paper electrophoresis as an analytical tool for the separation of alkaloids.

EXPERIMENTAL

The leaves of *D. stramonium* L., *D. stramonium* L. var. *inermis*, and *D. tatula* L. were harvested from 17-week-old plants that were in the flowering and fruiting stage of development. The leaves were air dried at room temperature and then reduced to a number 40 powder.

Determination of Total Alkaloids and Individual Alkaloids.—Ten-gram samples were extracted for total alkaloids by the official procedure in the assay of Stramonium (3). The total alkaloidal residue was dissolved in chloroform and made up quantitatively to a 5-ml. volume. Aliquots of the alkaloid-chloroform solution were assayed for total alkaloids by the Vitali-Morin colorimetric assay (4, 5). The results of the assay for total alkaloids are listed in Table I.

It was possible to separate the individual alkaloids by the procedure described by Drey and Foster (6) utilizing the technique of paper chromatography. Aliquots of the alkaloid-chloroform solution used for the total alkaloid assay were placed on paper strips for the paper chromatographic separation. By this procedure the alkaloids hyosycamine and scopolamine were separated into two distinct spots, hyoscyamine having an R_f of 0.65 and scopolamine an R_f of 0.85. The paper strips were cut so that the hyoscyamine was on one cut strip and the scopolamine on the other. The alkaloids were then eluted from each paper strip with alcohol. The amount of alkaloid present was then determined by the Vitali-Morin colorimetric assay. The results of the assays are listed in Table I.

Separation of Hyoscyamine and Scopolamine by Paper Electrophoresis.—For the separation of the alkaloids hyoscyamine and scopolamine by paper electrophoresis, the following materials were used: a power kit (Heath-Kit, Model PS-2); two carbon electrodes; two enameled pans; Whatman paper No. 1; two glass rods to support the paper; and a glass-enclosed aquarium tank.

Known samples of the alkaloids in chloroform were placed on strips of paper 40 cm. long and 3 cm. wide. These strips were soaked in a buffer solution and then placed in a horizontal position with each end hanging down over a glass rod. Each end extended into a buffer solution in which there was a carbon electrode. The alkaloids were placed near the positive pole on the horizontal portion of the paper strip. Different buffer solutions with *p*H values ranging from 4.2 to 10.5 were tried in order to find the optimum *p*H for the separation of the alkaloids. At completion of the run the strips were air-dried and then developed with 0.2 per cent iodine T.S. to detect the alkaloidal spots on the paper. The distance of migration was measured from the starting point of the alkaloid to the beginning of the alkaloidal spot at the completion of the run. The distance of migration of the alkaloids after two hours is shown in Table II and after four hours in Table III.

TABLE I.—RESULTS OF THE ALKALOIDAL ASSAYS

Specie of Datura Sample	Per cent Total Alkaloid[a]	Per cent Hyoscyamine[a]	Per cent Scopolamine[a]	Per cent Scopolamine Plus Hyoscyamine	S:H Ratio
Tatula	0.435	0.402	0.033	0.435	1/12.12
Stramonium	0.553	0.490	0.066	0.556	1/7.42
Stramonium var. *inermis*	0.417	0.379	0.039	0.417	1/9.72

[a] Each result is the average of five assays.

* Received April 23, 1955, from the college of Pharmacy, Ohio State University, Columbus, Ohio.

Abstracted from a dissertation presented to the Graduate School of The Ohio State University by Riad Ramadan Alami in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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TABLE II.—THE DISTANCE OF MIGRATION OF HYOSCYAMINE AND SCOPOLAMINE AT DIFFERENT *p*H VALUES WITH 480 VOLTS FOR A TWO-HOUR PERIOD

*p*H	Migration of Hyoscyamine, cm.	Migration of Scopolamine, cm.	Migration of an Equal Mixture of Hyoscyamine and Scopolamine cm.	cm.
4.2	12.36	12.04	12.04	12.04
7.4	12.00	11.00	11.50	11.50
8.5	11.90	7.70	12.20	12.20
9.5	13.30	4.80	13.30	4.90
10.5	15.50	6.90	15.30	6.90

TABLE III.—THE DISTANCE OF MIGRATION OF HYOSCYAMINE AND SCOPOLAMINE AT DIFFERENT *p*H VALUES WITH 480 VOLTS FOR A FOUR-HOUR PERIOD

*p*H	Migration of Hyoscyamine, cm.	Migration of Scopolamine, cm.	Migration of an Equal Mixture of Hyoscyamine and Scopolamine cm.	cm.
4.2	21.04	21.70	19.10	19.10
7.4	13.20	12.50	13.40	13.40
8.5	20.80	15.50	20.80	20.80
9.5	18.50	8.60	18.30	8.50
10.5	19.70	10.40	19.60	10.60

DISCUSSION

By noting the values of the total alkaloids listed in Table I it may be observed that the differences in the alkaloid data for the three Datura species are not great. *D. stramonium* had the greater percentage of alkaloids as well as a greater proportion of scopolamine as indicated by the scopalamine to hyoscyamine ratio listed in Table I.

Kariyone, *et al.* (7), were among the first investigators to use paper electrophoresis in relation to alkaloids. They reported the migration distance at certain voltage and certain periods of time for various organic bases among which was the alkaloid hyoscyamine.

The major advantage of paper electrophoresis over paper chromatography in the separation of hyoscyamine and scopolamine is that it is less time consuming. Two to four hours was sufficient for alkaloidal separation with paper electrophoresis, while paper chromatography required fifteen hours.

It may be noted in Tables II and III that the rate of migration of hyoscyamine was about the same at all *p*H values while the scopolamine migration rate changed markedly at *p*H 9.5. Thus a complete separation of the two alkaloids was obtained at *p*H 9.5. It is interesting to note that scopolamine was the leading spot in paper chromatography, but in paper electrophoresis it was the slower component.

It is felt that this process shows great promise as an analytical tool in plant drug investigation, and more work is being planned along this line.

SUMMARY AND CONCLUSIONS

1. Leaf samples of *Datura stramonium* L., *Datura stramonium* L. var. *inermis*, and *Datura tatula* L. obtained from the Medicinal Plant Garden of the College of Pharmacy of The Ohio State University were assayed for total alkaloids.

(*a*) *Datura stramonium* L. contained 0.553 per cent total alkaloids.

(*b*) *Datura stramonium* L. var. *inermis* contained 0.417 per cent total alkaloids.

(*c*) *Datura tatula* L. contained 0.435 per cent total alkaloids.

2. The alkaloids hyoscyamine and scopolamine were separated by paper chromatography, identified and assayed. The relative amounts of the two alkaloids were expressed by the scopolamine-hyoscyamine ratio.

(*a*) *Datura stramonium* L. had a S-H ratio of 1:7.42.

(*b*) *Datura stramonium* L. var. *inermis* had a S-H ratio of 1:9.72.

(*c*) *Datura tatula* L. had a S-H ratio of 1:12.12.

3. The alkaloids hyocyamine and scopolamine were separated also by paper electrophoresis. The separation was achieved at *p*H values of 9.5 and 10.5 after a two-hour period of electromigration.

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Book Notices

Substances Naturelles de Synthèse. Préparations et Méthods de Laboratoire. Vol. 10. LEON VELLUZ, Editor. Masson et C^ie^, Paris VI^e^, 1954. 201 pp. 16 x 22.5 cm. Illustrated. Price (paper) 2.300 fr. (cloth) 2.700 fr.

Volume X in this series of monographs prepared by A. Allais, J. Mathieu, A. Petit, P. Poirier, and L. Velluz presents, in the established style, methods of preparation for DL-orginine, axerophthol, L-carnosine, β-carotene, ergothioneine, D-glucosamine, DL-penicillamine, and retinene in the first part of the book. The second portion of the text gives a review of the methods of synthesis of peptides and includes a tabulated reference to the peptides. The synthesis of isoprenic chains is also discussed in this section. Intermediates in the peptide syntheses are discussed in a separate section. Tables on maximum allowable concentration of some noxious solvent vapors; flammability and auto-ignition of some solvent

vapors; solubility of some gases in common solvents; and stalagmometric constants of some solvents and concentrated solutions are given. A table of all compounds mentioned in volumes VI to X, inclusive, is appended.

The Human Adrenal Cortex, Vol. 8. Ciba Foundation (London) Colloquia on Endocrinology. G. E. W. WOLSTENHOLME, MARGARET P. CAMERON, and JOAN ETHERINGTON, Editors. Little, Brown and Company, Boston, 1955. xv + 665 pp. 14.5 x 21 cm. Price $10.

This book is a complete compilation of the 35 papers and the open discussions at the eighth symposium on endocrinology sponsored by the Ciba Foundation and held April 1954. Subjects of particular interest to pharmacists are: biological studies with aldosterone (electrocortin); variability of adrenocortical response to ACTH in different persons, and the influence of variations in administration of ACTH preparations on the level of 17-hydroxycorticosteroids in the blood; the reciprocal relationship between the thyroid and adrenocortical responses to stress; psychological responses to the administration of ACTH and cortisone; and adrenocortical function of combat infantrymen in Korea. A general index is appended. This volume continues the excellence of material and presentation established in the earlier publications of the Ciba Foundation.

Ageing—General Aspects. Vol. 1. Ciba Foundation (London) Colloquia on Ageing. G. E. W. WOLSTENHOLME, MARGARET P. CAMERON, and JOAN ETHERINGTON, Editors. Little, Brown and Company, Boston, 1955. xi + 255 pp. 14.5 x 21 cm. Price $6.75.

The first volume in this new series called "Colloquia in Ageing" is a compilation of the papers and discussions at the Ciba Foundation's symposium on the general aspects of aging held July 1954. Thirty-one participants from many countries contribute to the interesting material presented and developed in the discussions which cover mental and physiological aspects of the subject. A general discussion and an index are included.

Clinical Toxicology. By CLINTON J. THIENES and THOMAS J. HALEY. Lea & Febiger, Philadelphia, 1955. 457 pp. 13.5 x 20 cm. Price $6.50.

This excellent textbook and guide for the medical practitioner adheres closely to the toxicology of poisonous substances, and introduces related material only when it is necessary. The text material is arranged according to major toxic action and drugs which act strongly on more than one physiological system are discussed or mentioned under the different headings.

Of particular value to the physician or pharmacist is "An Outline of Symptom Diagnosis." The chemical tests given in this book are reliable because most have been used by the authors. New poisonous agents included in this edition are: chlorinated hydrocarbon and organic phosphate insecticides, antihistamines, beryllium, iron, sulfur dioxide, formaldehyde, fluoroacetate, new curariform drugs, ganglionic and autonomic depressants, cortisone, and many others. Many tests have been revised, spectrophotometric tests for barbiturates have been included, and suggested maximum safe concentrations of many industrial air pollutants are listed.

The thorough coverage of the subject in concise yet clear language in a very good layout with easily readable type makes this volume a most useful reference that should be available to all pharmacists and physicians at the student and practitioner levels.

Einführung in die Geschichte der deutschen Pharmazie. By GEORG URDANG and HANS DIECKMANN. Govi-Verlag G. m. b. H., Frankfurt a/M, 1954. 142 pp. 16.5 x 24.8 cm. Price DM 8.50.

This little book sets a high standard for both the writers of history and the craftsmen of printing. It offers an *Introduction to the History of German Pharmacy* in a broad sense, with only about half the main text (46 pp.) devoted to Germany *per se*. Other sections concisely discuss the pre-German antecedents (14 pp.); evolution of medicine and pharmacy in Europe, particularly the medical theories and materia medica (29 pp.); and international pharmaceutical cooperation (10 pp.).

It is largely an elegant distillate from the senior author's books, *Grundriss der Geschichte der deutschen Pharmazie* (1935; out of print) in collaboration with Alfred Adlung and *History of Pharmacy* (1940) with Edward Kremers. Without substituting for these earlier works, the new *Einführung* has its own appeal for all who read German.

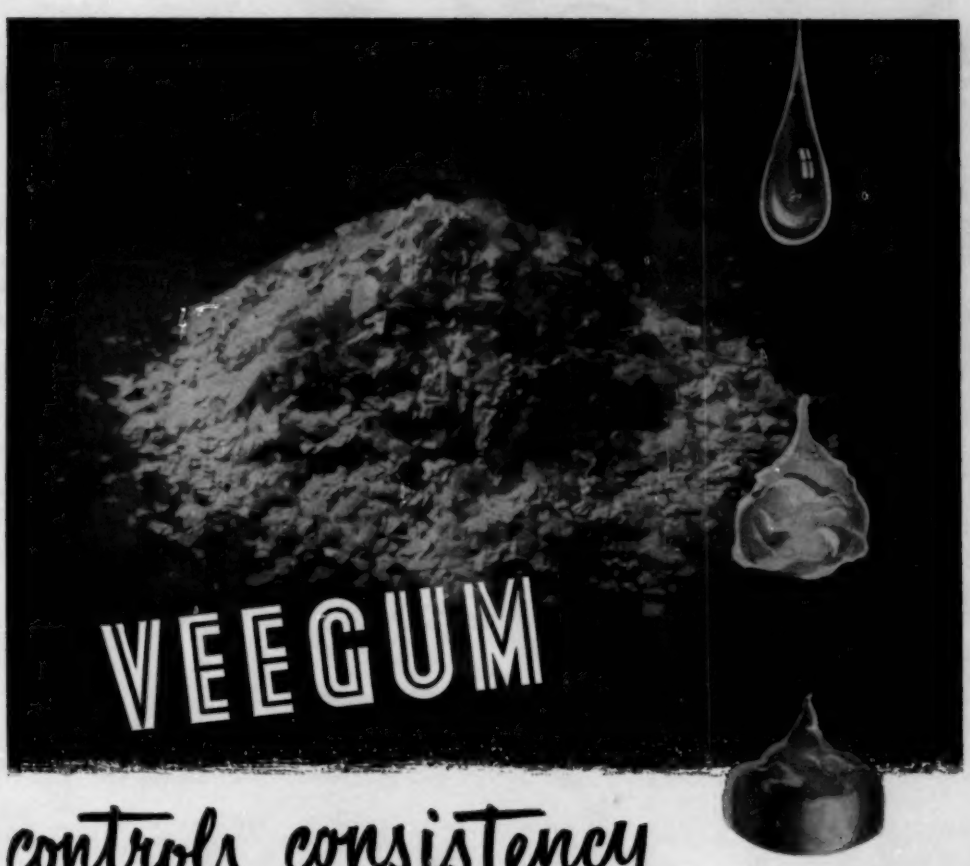
The book is handsomely bound and illustrated (8 plates), well indexed and documented.

The senior author, Georg Urdang, is Director of the American Institute of the History of Pharmacy and Professor-Emeritus of the University of Wisconsin. In the preface he has high praise for his collaborator, Hans Dieckmann, who was a post-doctoral fellow at the University of Wisconsin (history of pharmacy, 1953–1954) and is now on the staff of the World Health Organization in Geneva.—GLENN SONNEDECKER.

Biochemical Preparations. Vol. 4. W. W. WESTERFELD, Editor-in-chief. John Wiley & Sons, Inc., New York, 1955. vii + 108 pp. 16 x 23.5 cm. Price $3.75.

Volume 3 in this series was reviewed in THIS JOURNAL, **43,** 192(April 1954). Methods which have been checked and are considered to be reliable are given for the preparation of: α-D-galactose-1-phosphate; homogentisic acid; (dipalmitoleyl)-L-α-lecithin; α-lactalbumin; β-lactoglobulin; alcohol dehydrogenase; inorganic pyrophosphatase; carnosine; L-histidinol dihydrochloride; urocanic acid; N-acetyl imidazole; glycolaldehyde; sodium glyozylate monohydrate; α-D-glucose-1-phosphate; tetraacetyl-D ribofuranose; L-argininic acid; DL-methionine sulfoxide; DL-methionine sulfone and DL-ethionine sulfone; linoleic acid and methyl linoleate; and D-glutamic acid, alternative procedure.

The compounds of biochemical interest which have appeared in *Organic Syntheses* (through vol. 34) are listed in the front of this book. A subject index is appended.



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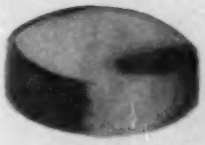

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